

## SELF-PROCESSING PLANTS AND PLANT PARTS

### Related Applications

This application is a continuation-in-part of U.S. Patent Application No. 10/228,063, filed August 27, 2002, which claims priority to Application Serial No. 60/315,281, filed August 27, 2001, each of which is herein incorporated by reference in their entirety.

### Field of the Invention

The present invention generally relates to the field of plant molecular biology, and more specifically, to the creation of plants that express a processing enzyme which provides a desired characteristic to the plant or products thereof.

### Background of the Invention

Enzymes are used to process a variety of agricultural products such as wood, fruits and vegetables, starches, juices, and the like. Typically, processing enzymes are produced and recovered on an industrial scale from various sources, such as microbial fermentation (*Bacillus*  $\alpha$ -amylase), or isolation from plants (coffee  $\beta$ -galactosidase or papain from plant parts). Enzyme preparations are used in different processing applications by mixing the enzyme and the substrate under the appropriate conditions of moisture, temperature, time, and mechanical mixing such that the enzymatic reaction is achieved in a commercially viable manner. The methods involve separate steps of enzyme production, manufacture of an enzyme preparation, mixing the enzyme and substrate, and subjecting the mixture to the appropriate conditions to facilitate the enzymatic reaction. A method that reduces or eliminates the time, energy, mixing, capital expenses, and/or enzyme production costs, or results in improved or novel products, would be useful and beneficial. One example of where such improvements are needed is in the area of corn milling.

Today corn is milled to obtain cornstarch and other corn-milling co-products such as corn gluten feed, corn gluten meal, and corn oil. The starch obtained from the process is often further processed into other products such as derivatized starches and sugars, or fermented to make a variety of products including alcohols or lactic acid. Processing of cornstarch often involves the use of enzymes, in particular, enzymes that hydrolyze and convert starch into fermentable sugars

or fructose ( $\alpha$ - and gluco-amylase,  $\alpha$ -glucosidase, glucose isomerase, and the like). The process used commercially today is capital intensive as construction of very large mills is required to process corn on scales required for reasonable cost-effectiveness. In addition the process requires the separate manufacture of starch-hydrolyzing or modifying enzymes and then the machinery to mix the enzyme and substrate to produce the hydrolyzed starch products.

The process of starch recovery from corn grain is well known and involves a wet-milling process. Corn wet-milling includes the steps of steeping the corn kernel, grinding the corn kernel and separating the components of the kernel. The kernels are steeped in a steep tank with a countercurrent flow of water at about 120° F and the kernels remain in the steep tank for 24 to 48 hours. This steepwater typically contains sulfur dioxide at a concentration of about 0.2% by weight. Sulfur dioxide is employed in the process to help reduce microbial growth and also to reduce disulfide bonds in endosperm proteins to facilitate more efficient starch-protein separation. Normally, about 0.59 gallons of steepwater is used per bushel of corn. The steepwater is considered waste and often contains undesirable levels of residual sulfur dioxide.

The steeped kernels are then dewatered and subjected to sets of attrition type mills. The first set of attrition type mills rupture the kernels releasing the germ from the rest of the kernel. A commercial attrition type mill suitable for the wet milling business is sold under the brand name Bauer. Centrifugation is used to separate the germ from the rest of the kernel. A typical commercial centrifugation separator is the Merco centrifugal separator. Attrition mills and centrifugal separators are large expensive items that use energy to operate.

In the next step of the process, the remaining kernel components including the starch, hull, fiber, and gluten are subjected to another set of attrition mills and passed through a set of wash screens to separate the fiber components from the starch and gluten (endosperm protein). The starch and gluten pass through the screens while the fiber does not. Centrifugation or a third grind followed by centrifugation is used to separate the starch from the endosperm protein. Centrifugation produces a starch slurry which is dewatered, then washed with fresh water and dried to about 12% moisture. The substantially pure starch is typically further processed by the use of enzymes.

The separation of starch from the other components of the grain is performed because removing the seed coat, embryo and endosperm proteins allows one to efficiently contact the starch with processing enzymes, and the resulting hydrolysis products are relatively free from contaminants from the other kernel components. Separation also ensures that other components of the grain are effectively recovered and can be subsequently sold as co-products to increase the revenues from the mill.

After the starch is recovered from the wet-milling process it typically undergoes the processing steps of gelatinization, liquefaction and dextrinization for maltodextrin production, and subsequent steps of saccharification, isomerization and refining for the production of glucose, maltose and fructose.

Gelatinization is employed in the hydrolysis of starch because currently available enzymes cannot rapidly hydrolyze crystalline starch. To make the starch available to the hydrolytic enzymes, the starch is typically made into a slurry with water (20-40% dry solids) and heated at the appropriate gelling temperature. For cornstarch this temperature is between 105-110° C. The gelatinized starch is typically very viscous and is therefore thinned in the next step called liquefaction. Liquefaction breaks some of the bonds between the glucose molecules of the starch and is accomplished enzymatically or through the use of acid. Heat-stable endo  $\alpha$ -amylase enzymes are used in this step, and in the subsequent step of dextrinization. The extent of hydrolysis is controlled in the dextrinization step to yield hydrolysis products of the desired percentage of dextrose.

Further hydrolysis of the dextrin products from the liquefaction step is carried out by a number of different exo-amylases and debranching enzymes, depending on the products that are desired. And finally if fructose is desired then immobilized glucose isomerase enzyme is typically employed to convert glucose into fructose.

Dry-mill processes of making fermentable sugars (and then ethanol, for example) from cornstarch facilitate efficient contacting of exogenous enzymes with starch. These processes are less capital intensive than wet-milling but significant cost advantages are still desirable, as often the co-products derived from these processes are not as valuable as those derived from wet-milling. For example, in dry milling corn, the kernel is ground into a powder to facilitate

efficient contact of starch by degrading enzymes. After enzyme hydrolysis of the corn flour the residual solids have some feed value as they contain proteins and some other components. Eckhoff recently described the potential for improvements and the relevant issues related to dry milling in a paper entitled "Fermentation and costs of fuel ethanol from corn with quick-germ process" (Appl. Biochem. Biotechnol., 94: 41 (2001)). The "quick germ" method allows for the separation of the oil-rich germ from the starch using a reduced steeping time.

One example where the regulation and/or level of endogenous processing enzymes in a plant can result in a desirable product is sweet corn. Typical sweet corn varieties are distinguished from field corn varieties by the fact that sweet corn is not capable of normal levels of starch biosynthesis. Genetic mutations in the genes encoding enzymes involved in starch biosynthesis are typically employed in sweet corn varieties to limit starch biosynthesis. Such mutations are in the genes encoding starch synthases and ADP-glucose pyrophosphorylases (such as the sugary and super-sweet mutations). Fructose, glucose and sucrose, which are the simple sugars necessary for producing the palatable sweetness that consumers of edible fresh corn desire, accumulate in the developing endosperm of such mutants. However, if the level of starch accumulation is too high, such as when the corn is left to mature for too long (late harvest) or the corn is stored for an excessive period before it is consumed, the product loses sweetness and takes on a starchy taste and mouthfeel. The harvest window for sweet corn is therefore quite narrow, and shelf-life is limited.

Another significant drawback to the farmer who plants sweet corn varieties is that the usefulness of these varieties is limited exclusively to edible food. If a farmer wanted to forego harvesting his sweet corn for use as edible food during seed development, the crop would be essentially a loss. The grain yield and quality of sweet corn is poor for two fundamental reasons. The first reason is that mutations in the starch biosynthesis pathway cripple the starch biosynthetic machinery and the grains do not fill out completely, causing the yield and quality to be compromised. Secondly, due to the high levels of sugars present in the grain and the inability to sequester these sugars as starch, the overall sink strength of the seed is reduced, which exacerbates the reduction of nutrient storage in the grain. The endosperms of sweet corn variety seeds are shrunken and collapsed, do not undergo proper desiccation, and are susceptible to diseases. The poor quality of the sweet corn grain has further agronomic implications; as poor



seed viability, poor germination, seedling disease susceptibility, and poor early seedling vigor result from the combination of factors caused by inadequate starch accumulation. Thus, the poor quality issues of sweet corn impact the consumer, farmer/grower, distributor, and seed producer.

Thus, for dry-milling, there is a need for a method which improves the efficiency of the process and/or increases the value of the co-products. For wet-milling, there is a need for a method of processing starch that does not require the equipment necessary for prolonged steeping, grinding, milling, and/or separating the components of the kernel. For example, there is a need to modify or eliminate the steeping step in wet milling as this would reduce the amount of waste water requiring disposal, thereby saving energy and time, and increasing mill capacity (kernels would spend less time in steep tanks). There is also a need to eliminate or improve the process of separating the starch-containing endosperm from the embryo.

#### Summary of the Invention

The present invention is directed to self-processing plants and plant parts and methods of using the same. The self-processing plant and plant parts of the present invention are capable of expressing and activating enzyme(s) (mesophilic, thermophilic, and/or hyperthermophilic). Upon activation of the enzyme(s) (mesophilic, thermophilic, or hyperthermophilic) the plant or plant part is capable of self-processing the substrate upon which it acts to obtain the desired result.

The present invention is directed to an isolated polynucleotide a) comprising SEQ ID NO: 2, 4, 6, 9, 19, 21, 25, 37, 39, 41, 43, 46, 48, 50, 52, or 59 or the complement thereof, or a polynucleotide which hybridizes to the complement of any one of SEQ ID NO: 2, 4, 6, 9, 19, 21, 25, 37, 39, 41, 43, 46, 48, 50, 52, or 59 under low stringency hybridization conditions and encodes a polypeptide having  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucose isomerase, or glucoamylase activity or b) encoding a polypeptide comprising SEQ ID NO: 10, 13, 14, 15, 16, 18, 20, 24, 26, 27, 28, 29, 30, 33, 34, 35, 36, 38, 40, 42, 44, 45, 47, 49, or 51 or an enzymatically active fragment thereof. Preferably, the isolated polynucleotide encodes a fusion polypeptide comprising a first polypeptide and a second peptide, wherein said first polypeptide has  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucose isomerase, or glucoamylase activity. Most preferably, the second peptide comprises a signal sequence peptide, which may target the first

polypeptide to a vacuole, endoplasmic reticulum, chloroplast, starch granule, seed or cell wall of a plant. For example, the signal sequence may be an N-terminal signal sequence from *waxy*, an N-terminal signal sequence from  $\gamma$ -zein, a starch binding domain, or a C-terminal starch binding domain. Polynucleotides that hybridize to the complement of any one of SEQ ID NO: 2, 9, or 52 under low stringency hybridization conditions and encodes a polypeptide having  $\alpha$ -amylase activity; to the complement of SEQ ID NO: 4 or 25 under low stringency hybridization conditions and encodes a polypeptide having pullulanase activity; to the complement of SEQ ID NO: 6 and encodes a polypeptide having  $\alpha$ -glucosidase activity; to the complement of any one of SEQ ID NO: 19, 21, 37, 39, 41, or 43 under low stringency hybridization conditions and encodes a polypeptide having glucose isomerase activity; to the complement of any one of SEQ ID NO: 46, 48, 50, or 59 under low stringency hybridization conditions and encodes a polypeptide having glucoamylase activity are further encompassed.

The present invention is also directed to an isolated polynucleotide a) comprising SEQ ID NO: 61, 63, 65, 79, 81, 83, 85, 87, 89, 91, 93, 94, 95, 96, 97, 99, 108, and 110 or the complement thereof, or a polynucleotide which hybridizes to the complement of any one of SEQ ID NO: 61, 63, 65, 79, 81, 83, 85, 87, 89, 91, 93, 94, 95, 96, 97, 99, 108, or 110 under low stringency hybridization conditions and encodes a polypeptide having xylanase, cellulase, glucanase, beta glucosidase, esterase or phytase activity b) encoding a polypeptide comprising SEQ ID NO: 62, 64, 66, 70, 80, 82, 84, 86, 88, 90, 92, 109, or 111 or an enzymatically active fragment thereof. The isolated polynucleotide may encode a fusion polypeptide comprising a first polypeptide and a second peptide, wherein said first polypeptide has xylanase, cellulase, glucanase, beta glucosidase, protease, or phytase activity. The second peptide may comprises a signal sequence peptide, which may target the first polypeptide to a vacuole, endoplasmic reticulum, chloroplast, starch granule, seed or cell wall of a plant. For example, the signal sequence may be an N-terminal signal sequence from *waxy*, an N-terminal signal sequence from  $\gamma$ -zein, a starch binding domain, or a C-terminal starch binding domain.

Exemplary xylanases provided and useful in the invention include those encoded by SEQ ID NO: 61, 63, or 65. An exemplary protease, namely bromelain, encoded by SEQ ID NO: 69 is also provided. Exemplary cellulases include cellobiohydrolase I and II as provided herein and

encoded by SEQ ID NO: 79, 81, 93, and 94. An exemplary glucanase is provided as 6GP1 described herein encoded by SEQ ID NO: 85. Exemplary beta glucosidases include beta glucosidase 2 and D, as described herein and encoded by SEQ ID NO: 96 and 97. An exemplary esterase is also provided, namely ferulic acid esterase as encoded by SEQ ID NO: 99. And, an exemplary phytase, Nov9X as encoded by SEQ ID NO: 109-112 is also provided.

Also included are expression cassettes comprising a polynucleotide a) having SEQ ID NO: 2, 4, 6, 9, 19, 21, 25, 37, 39, 41, 43, 46, 48, 50, 52, or 59 or the complement thereof, or a polynucleotide which hybridizes to the complement of any one of SEQ ID NO: 2, 4, 6, 9, 19, 21, 25, 37, 39, 41, 43, 46, 48, 50, 52, or 59 or under low stringency hybridization conditions and encodes a polypeptide having  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucose isomerase, or glucoamylase activity or b) encoding a polypeptide comprising SEQ ID NO: 10, 13, 14, 15, 16, 18, 20, 24, 26, 27, 28, 29, 30, 33, 34, 35, 36, 38, 40, 42, 44, 45, 47, 49, or 51, or an enzymatically active fragment thereof. The expression cassette further comprises a promoter operably linked to the polynucleotide, such as an inducible promoter, tissue-specific promoter, or preferably an endosperm-specific promoter. Preferably, the endosperm-specific promoter is a maize  $\gamma$ -zein promoter or a maize ADP-gpp promoter or a maize Q promoter or a rice glutelin-1 promoter. In a preferred embodiment, the promoter comprises SEQ ID NO: 11 or SEQ ID NO: 12 or SEQ ID NO: 67 or SEQ ID NO: 98. Moreover, in another preferred embodiment the polynucleotide is oriented in sense orientation relative to the promoter. The expression cassette of the present invention may further encode a signal sequence which is operably linked to the polypeptide encoded by the polynucleotide. The signal sequence preferably targets the operably linked polypeptide to a vacuole, endoplasmic reticulum, chloroplast, starch granule, seed or cell wall of a plant. The signal sequences include an N-terminal signal sequence from *waxy*, an N-terminal signal sequence from  $\gamma$ -zein, or a starch binding domain.

Moreover, an expression cassette comprising a polynucleotide a) having SEQ ID NO: 61, 63, 65, 79, 81, 83, 85, 87, 89, 91, 93, 94, 95, 96, 97, 99, 108, and 110 or the complement thereof, or a polynucleotide which hybridizes to the complement of any one of SEQ ID NO: 61, 63, 65, 79, 81, 83, 85, 87, 89, 91, 93, 94, 95, 96, 97, 99, 108, and 110 or under low stringency hybridization conditions and encodes a polypeptide having xylanase, cellulase, glucanase, beta

glucosidase, esterase or phytase activity or b) encoding a polypeptide comprising SEQ ID NO: 62, 64, 66, 70, 80, 82, 84, 86, 88, 90, 92, 109, or 111, or an enzymatically active fragment thereof. The expression cassette further comprises a promoter operably linked to the polynucleotide, such as an inducible promoter, tissue-specific promoter, or preferably an endosperm-specific promoter. The endosperm-specific promoter may be a maize  $\gamma$ -zein promoter or a maize ADP-gpp promoter or a maize Q promoter promoter or a rice glutelin-1 promoter. In an embodiment, the promoter comprises SEQ ID NO: 11 or SEQ ID NO: 12 or SEQ ID NO: 67 or SEQ ID NO: 98. Moreover, in another embodiment the polynucleotide is oriented in sense orientation relative to the promoter. The expression cassette of the present invention may further encode a signal sequence which is operably linked to the polypeptide encoded by the polynucleotide. The signal sequence preferably targets the operably linked polypeptide to a vacuole, endoplasmic reticulum, chloroplast, starch granule, seed or cell wall of a plant. The signal sequences include an N-terminal signal sequence from *waxy*, an N-terminal signal sequence from  $\gamma$ -zein, or a starch binding domain.

The present invention is further directed to a vector or cell comprising the expression cassettes of the present invention. The cell may be selected from the group consisting of an *Agrobacterium*, a monocot cell, a dicot cell, a Liliopsida cell, a Panicoideae cell, a maize cell, and a cereal cell, such as a rice cell.

Moreover, the present invention encompasses a plant stably transformed with the vectors of the present invention. A plant stably transformed with a vector comprising an  $\alpha$ -amylase having an amino acid sequence of any of SEQ ID NO: 1, 10, 13, 14, 15, 16, 33, 35 or 88 or encoded by a polynucleotide comprising any of SEQ ID NO: 2, 9, or 87 is provided.

In another embodiment, a plant stably transformed with a vector comprising a pullulanase having an amino acid sequence of any of SEQ ID NO: 24 or 34, or encoded by a polynucleotide comprising any of SEQ ID NO: 4 or 25 is provided. A plant stably transformed with a vector comprising an  $\alpha$ -glucosidase having an amino acid sequence of any of SEQ ID NO: 26 or 27, or encoded by a polynucleotide comprising SEQ ID NO: 6 is further provided. A plant stably transformed with a vector comprising a glucose isomerase having an amino acid sequence of any of SEQ ID NO: 18, 20, 28, 29, 30, 38, 40, 42, or 44, or encoded by a polynucleotide

comprising any of SEQ ID NO:19, 21, 37, 39, 41, or 43 is further described herein. In another embodiment, a plant stably transformed with a vector comprising a glucose amylase having an amino acid sequence of any of SEQ ID NO: 45, 47, or 49, or encoded by a polynucleotide comprising any of SEQ ID NO:46, 48, 50, or 59 is described.

An additional embodiment provides a plant stably transformed with a vector comprising a xylanase having an amino acid sequence of any of SEQ ID NO: 62, 64 or 66, or encoded by a polynucleotide comprising any of SEQ ID NO: 61, 63, or 65. A plant stably transformed with a vector comprising a protease is also provided. The protease may be bromelain having an amino acid sequence as set forth in SEQ ID NO: 70, or encoded by a polynucleotide having SEQ ID NO: 69. In another embodiment, a plant stably transformed with a vector comprising a cellulase is provided. The cellulase may be a cellobiohydrolase encoded by a polynucleotide comprising any of SEQ ID NO: 79, 80, 81, 82, 93 or 94.

An additional embodiment provides a plant stably transformed with a vector comprising a glucanase, such as an endoglucanase. The endoglucanase may be endoglucanase I which has an amino acid sequence as in SEQ ID NO: 84, or encoded by a polynucleotide comprising SEQ ID NO: 83. A plant stably transformed with a vector comprising a beta glucosidase is also provided. The beta glucosidase is may be beta glucosidase 2 or beta glucosidase D, which have an amino acid sequence set forth in SEQ ID NO: 90 or 92, or encoded by a polynucleotide having SEQ ID NO: 89 or 91. In another embodiment, a plant stably transformed with a vector comprising an esterase is provided. The esterase may be a ferulic acid esterase encoded by a polynucleotide comprising SEQ ID NO: 99.

Plant products, such as seed, fruit or grain from the stably transformed plants of the present invention are further provided.

In another embodiment, the invention is directed to a transformed plant, the genome of which is augmented with a recombinant polynucleotide encoding at least one processing enzyme operably linked to a promoter sequence, the sequence of which polynucleotide is optimized for expression in the plant. The plant may be a monocot, such as maize or rice, or a dicot. The plant may be a cereal plant or a commercially grown plant. The processing enzyme is selected from the group consisting of an  $\alpha$ -amylase, glucoamylase, glucose isomerase, glucanase,  $\beta$ -



amylase,  $\alpha$ -glucosidase, isoamylase, pullulanase, neo-pullulanase, iso-pullulanase, amylopullulanase, cellulase, exo-1,4- $\beta$ -cellobiohydrolase, exo-1,3- $\beta$ -D-glucanase,  $\beta$ -glucosidase, endoglucanase, L-arabinase,  $\alpha$ -arabinosidase, galactanase, galactosidase, mannanase, mannosidase, xylanase, xylosidase, protease, glucanase, xylanase, , esterase, phytase, and lipase. The processing enzyme is a starch-processing enzyme selected from the group consisting of  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\beta$ -amylase,  $\alpha$ -glucosidase, isoamylase, pullulanase, neo-pullulanase, iso-pullulanase, and amylopullulanase. The enzyme may be selected from  $\alpha$ -amylase, glucoamylase, glucose isomerase, glucose isomerase,  $\alpha$ -glucosidase, and pullulanase. The processing enzyme may be hyperthermophilic. In accordance with this aspect of the invention, the enzyme may be a non-starch degrading enzyme selected from the group consisting of protease, glucanase, xylanase, esterase, phytase, cellulase, beta glucosidase, and lipase. Such enzymes may be hyperthermophilic. In an embodiment, the enzyme accumulates in the vacuole, endoplasmic reticulum, chloroplast, starch granule, seed or cell wall of a plant. Moreover, in another embodiment, the genome of plant may be further augmented with a second recombinant polynucleotide comprising a non-hyperthermophilic enzyme.

In another aspect of the invention, provided is a transformed plant, the genome of which is augmented with a recombinant polynucleotide encoding at least one processing enzyme selected from the group consisting of  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\alpha$ -glucosidase, pullulanase, xylanase, cellulase, protease, glucanase, beta glucosidase, esterase, phytase or lipase operably linked to a promoter sequence, the sequence of which polynucleotide is optimized for expression in the plant.

Another embodiment is directed to a transformed maize plant, the genome of which is augmented with a recombinant polynucleotide encoding at least one processing enzyme selected from the group consisting of  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\alpha$ -glucosidase, pullulanase, xylanase, cellulase, protease, glucanase, phytase, beta glucosidase, esterase, or lipase operably linked to a promoter sequence, the sequence of which polynucleotide is optimized for expression in the maize plant.

A transformed plant, the genome of which is augmented with a recombinant polynucleotide having SEQ ID NO: 83 operably linked to a promoter and to a signal sequence is

provided. Additionally, a transformed plant, the genome of which is augmented with a recombinant polynucleotide having the SEQ ID NO: 93 or 94 operably linked to a promoter and to a signal sequence is described. In another embodiment, a transformed plant, the genome of which is augmented with a recombinant polynucleotide having SEQ ID NO: 95, operably linked to a promoter and to a signal sequence. Moreover, a transformed plant, the genome of which is augmented with a recombinant polynucleotide having SEQ ID NO: 96 is described. Also described is a transformed plant, the genome of which is augmented with a recombinant polynucleotide having SEQ ID NO: 97. Also described is a transformed plant, the genome of which is augmented with a recombinant polypeptide having SEQ ID NO: 99.

Products of the transformed plants are further envisioned herein. The product for example, include seed, fruit, or grain. The product may alternatively be the processing enzyme, starch or sugar.

A plant obtained from a stably transformed plant of the present invention is further described. In this aspect, the plant may be a hybrid plant or an inbred plant.

A starch composition is a further embodiment of the invention comprising at least one processing enzyme which is a protease, glucanase, or esterase.

Grain is another embodiment of the invention comprising at least one processing enzyme, which is an  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucoamylase, glucose isomerase, xylanase, cellulase, glucanase, beta glucosidase, esterase, protease, lipase or phytase.

In another embodiment, a method of preparing starch granules, comprising treating grain which comprises at least one non-starch processing enzyme under conditions which activate the at least one enzyme, yielding a mixture comprising starch granules and non-starch degradation products, wherein the grain is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one enzyme; and separating starch granules from the mixture is provided. Therein, the enzyme may be a protease, glucanase, xylanase, phytase, lipase, beta glucosidase, cellulase or esterase. Moreover, the enzyme is preferably hyperthermophilic. The grain may be cracked grain and/or may be treated under low or high moisture conditions. Alternatively, the grain may be treated with sulfur dioxide. The present invention may further comprise separating non-starch products from the mixture. The starch products and non-starch products obtained by this method are further described.

In yet another embodiment, a method to produce hypersweet corn comprising treating transformed corn or a part thereof, the genome of which is augmented with and expresses in the endosperm an expression cassette encoding at least one starch-degrading or starch-isomerizing enzyme, under conditions which activate the at least one enzyme so as to convert polysaccharides in the corn into sugar, yielding hypersweet corn is provided. The expression cassette may further comprises a promoter operably linked to the polynucleotide encoding the enzyme. The promoter may be a constitutive promoter, seed-specific promoter, or endosperm-specific promoter, for example. The enzyme may be hyperthermophilic and may be an  $\alpha$ -amylase. The expression cassette used herein may further comprise a polynucleotide which encodes a signal sequence operably linked to the at least one enzyme. The signal sequence may direct the enzyme to the apoplast or the endoplasmic reticulum, for example. The enzyme comprises any one of SEQ ID NO: 13, 14, 15, 16, 33, or 35. The enzyme may also comprise SEQ ID NO: 87.

In a most preferred embodiment, a method of producing hypersweet corn comprising treating transformed corn or a part thereof, the genome of which is augmented with and expresses in the endosperm an expression cassette encoding an  $\alpha$ -amylase, under conditions which activate the at least one enzyme so as to convert polysaccharides in the corn into sugar, yielding hypersweet corn is described. The enzyme may be hyperthermophilic and the hyperthermophilic  $\alpha$ -amylase may comprise the amino acid sequence of any of SEQ ID NO: 10, 13, 14, 15, 16, 33, or 35, or an enzymatically active fragment thereof having  $\alpha$ -amylase activity. The enzyme comprise SEQ ID NO: 87.

A method to prepare a solution of hydrolyzed starch product comprising; treating a plant part comprising starch granules and at least one processing enzyme under conditions which activate the at least one enzyme thereby processing the starch granules to form an aqueous solution comprising hydrolyzed starch product, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one starch processing enzyme; and collecting the aqueous solution comprising the hydrolyzed starch product is described herein. The hydrolyzed starch product may comprise a dextrin, maltooligosaccharide, glucose and/or mixtures thereof. The enzyme may be  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase,

amylopullulanase, glucose isomerase, or any combination thereof. Moreover, the enzyme may be hyperthermophilic. In another aspect, the genome of the plant part may be further augmented with an expression cassette encoding a non-hyperthermophilic starch processing enzyme. The non-hyperthermophilic starch processing enzyme may be selected from the group consisting of amylase, glucoamylase,  $\alpha$ -glucosidase, pullulanase, glucose isomerase, or a combination thereof. In yet another aspect, the processing enzyme is preferably expressed in the endosperm. The plant part may be grain, and from corn, wheat, barley, rye, oat, sugar cane or rice. The at least one processing enzyme is operably linked to a promoter and to a signal sequence that targets the enzyme to the starch granule or the endoplasmic reticulum, or to the cell wall. The method may further comprise isolating the hydrolyzed starch product and/or fermenting the hydrolyzed starch product.

In another aspect of the invention, a method of preparing hydrolyzed starch product comprising treating a plant part comprising starch granules and at least one starch processing enzyme under conditions which activate the at least one enzyme thereby processing the starch granules to form an aqueous solution comprising a hydrolyzed starch product, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding at least one  $\alpha$ -amylase; and collecting the aqueous solution comprising hydrolyzed starch product is described. The  $\alpha$ -amylase may be hyperthermophilic and the hyperthermophilic  $\alpha$ -amylase comprises the amino acid sequence of any of SEQ ID NO: 1, 10, 13, 14, 15, 16, 33, or 35, or an active fragment thereof having  $\alpha$ -amylase activity. The expression cassette may comprise a polynucleotide selected from any of SEQ ID NO: 2, 9, 46, or 52, a complement thereof, or a polynucleotide that hybridizes to any of SEQ ID NO: 2, 9, 46, or 52 under low stringency hybridization conditions and encodes a polypeptide having  $\alpha$ -amylase activity. Moreover, the invention further provides for the genome of the transformed plant further comprising a polynucleotide encoding a non-thermophilic starch-processing enzyme. Alternatively, the plant part may be treated with a non-hyperthermophilic starch-processing enzyme.

The present invention is further directed to a transformed plant part comprising at least one starch-processing enzyme present in the cells of the plant, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette

encoding the at least one starch processing enzyme. Preferably, the enzyme is a starch-processing enzyme selected from the group consisting of  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\beta$ -amylase,  $\alpha$ -glucosidase, isoamylase, pullulanase, neo-pullulanase, iso-pullulanase, and amylopullulanase. Moreover, the enzyme may be hyperthermophilic. The plant may be any plant, such as corn or rice for example.

Another embodiment of the invention is a transformed plant part comprising at least one non-starch processing enzyme present in the cell wall or the cells of the plant, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one non-starch processing enzyme or at least one non-starch polysaccharide processing enzyme. The enzyme may be hyperthermophilic. Moreover, the non-starch processing enzyme may be a protease, glucanase, xylanase, esterase, phytase, beta glucosidase, cellulase or lipase. The plant part can be any plant part, but preferably is an ear, seed, fruit, grain, stover, chaff, or bagasse.

The present invention is also directed to transformed plant parts. For example, a transformed plant part comprising an  $\alpha$ -amylase having an amino acid sequence of any of SEQ ID NO: 1, 10, 13, 14, 15, 16, 33, or 35, or encoded by a polynucleotide comprising any of SEQ ID NO: 2, 9, 46, or 52, a transformed plant part comprising an  $\alpha$ -glucosidase having an amino acid sequence of any of SEQ ID NO: 5, 26 or 27, or encoded by a polynucleotide comprising SEQ ID NO:6, a transformed plant part comprising a glucose isomerase having the amino acid sequence of any one of SEQ ID NO: 28, 29, 30, 38, 40, 42, or 44, or encoded by a polynucleotide comprising any one of SEQ ID NO: 19, 21, 37, 39, 41, or 43, a transformed plant part comprising a glucoamylase having the amino acid sequence of SEQ ID NO:45 or SEQ ID NO:47, or SEQ ID NO:49, or encoded by a polynucleotide comprising any of SEQ ID NO: 46, 48, 50, or 59, and a transformed plant part comprising a pullulanase encoded by a polynucleotide comprising any of SEQ ID NO: 4 or 25 are described.

The present invention is also directed to transformed plant parts. For example, a transformed plant part comprising a xylanase having an amino acid sequence of any of SEQ ID NO: 62, 64 or 66, or encoded by a polynucleotide comprising any of SEQ ID NO: 61, 63, or 65.



A transformed plant part comprising a protease is also provided. The protease may be bromelain having an amino acid sequence as set forth in SEQ ID NO: 70, or encoded by a polynucleotide having SEQ ID NO: 69. In another embodiment, a transformed plant part comprising a cellulase is provided. The cellulase may be a cellobiohydrolase encoded by a polynucleotide comprising any of SEQ ID NO: 79, 80, 81, 82, 93 or 94.

An additional embodiment provides a transformed plant part a glucanase, such as an endoglucanase. The endoglucanase may be endoglucanase I which has an amino acid sequence as in SEQ ID NO: 84, or encoded by a polynucleotide comprising SEQ ID NO: 83. A transformed plant part comprising a beta glucosidase is also provided. The beta glucosidase is may be beta glucosidase 2 or beta glucosidase D, which have an amino acid sequence set forth in SEQ ID NO: 90 or 92, or encoded by a polynucleotide having SEQ ID NO: 89 or 91. In another embodiment, a transformed plant part comprising an esterase is provided. The esterase may be a ferulic acid esterase encoded by a polynucleotide comprising SEQ ID NO: 99.

Another embodiment is a method of converting starch in the transformed plant part comprising activating the starch processing enzyme contained therein. The starch, dextrin, maltooligosaccharide or sugar produced according to this method is further described.

The present invention further describes a method of using a transformed plant part comprising at least one non-starch processing enzyme in the cell wall or the cell of the plant part, comprising treating a transformed plant part comprising at least one non-starch polysaccharide processing enzyme under conditions so as to activate the at least one enzyme thereby digesting non-starch polysaccharide to form an aqueous solution comprising oligosaccharide and/or sugars, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one non-starch polysaccharide processing enzyme; and collecting the aqueous solution comprising the oligosaccharides and/or sugars. The non-starch polysaccharide processing enzyme may be hyperthermophilic.

A method of using transformed seeds comprising at least one processing enzyme, comprising treating transformed seeds which comprise at least one protease or lipase under conditions so as the activate the at least one enzyme yielding an aqueous mixture comprising amino acids and fatty acids, wherein the seed is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one enzyme; and

collecting the aqueous mixture. The amino acids, fatty acids or both are preferably isolated. The at least one protease or lipase may be hyperthermophilic.

A method to prepare ethanol comprising treating a plant part comprising at least one polysaccharide processing enzyme under conditions to activate the at least one enzyme thereby digesting polysaccharide to form oligosaccharide or fermentable sugar, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one polysaccharide processing enzyme; and incubating the fermentable sugar under conditions that promote the conversion of the fermentable sugar or oligosaccharide into ethanol. The

plant part may be a grain, fruit, seed, stalks, wood, vegetable or root. The plant part may be obtained from a plant selected from the group consisting of oats, barley, wheat, berry, grapes, rye, corn, rice, potato, sugar beet, sugar cane, pineapple, grasses and trees.

In another preferred embodiment, the polysaccharide processing enzyme is  $\alpha$ -amylase, glucoamylase,  $\alpha$ -glucosidase, glucose isomerase, pullulanase, or a combination thereof.

A method to prepare ethanol comprising treating a plant part comprising at least one enzyme selected from the group consisting of  $\alpha$ -amylase, glucoamylase,  $\alpha$ -glucosidase, glucose isomerase, or pullulanase, or a combination thereof, with heat for an amount of time and under conditions to activate the at least one enzyme thereby digesting polysaccharide to form fermentable sugar, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one enzyme; and incubating the fermentable sugar under conditions that promote the conversion of the fermentable sugar into ethanol is provided. The at least one enzyme may be hyperthermophilic or mesophilic.

In another embodiment, a method to prepare ethanol comprising treating a plant part comprising at least one non-starch processing enzyme under conditions to activate the at least one enzyme thereby digesting non-starch polysaccharide to oligosaccharide and fermentable sugar, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one enzyme; and incubating the fermentable sugar under conditions that promote the conversion of the fermentable sugar into

ethanol is provided. The non-starch processing enzyme may be a xylanase, cellulase, glucanase, beta glucosidase, protease, esterase, lipase or phytase.

A method to prepare ethanol comprising treating a plant part comprising at least one enzyme selected from the group consisting of  $\alpha$ -amylase, glucoamylase,  $\alpha$ -glucosidase, glucose isomerase, or pullulanase, or a combination thereof, under conditions to activate the at least one enzyme thereby digesting polysaccharide to form fermentable sugar, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one enzyme; and incubating the fermentable sugar under conditions that promote the conversion of the fermentable sugar into ethanol is further provided. The enzyme may be hyperthermophilic.

Moreover, a method to produce a sweetened farinaceous food product without adding additional sweetener comprising treating a plant part comprising at least one starch processing enzyme under conditions which activate the at least one enzyme, thereby processing starch granules in the plant part to sugars so as to form a sweetened product, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one enzyme; and processing the sweetened product into a farinaceous food product is described. The farinaceous food product may be formed from the sweetened product and water. Moreover, the farinaceous food product may contain malt, flavorings, vitamins, minerals, coloring agents or any combination thereof. The at least one enzyme may be hyperthermophilic. The enzyme may be selected from  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase, glucose isomerase, or any combination thereof. The plant may further be selected from the group consisting of soybean, rye, oats, barley, wheat, corn, rice and sugar cane. The farinaceous food product may be a cereal food, a breakfast food, a ready to eat food, or a baked food. The processing may include baking, boiling, heating, steaming, electrical discharge or any combination thereof.

The present invention is further directed to a method to sweeten a starch-containing product without adding sweetener comprising treating starch comprising at least one starch processing enzyme under conditions to activate the at least one enzyme thereby digesting the starch to form a sugar to form sweetened starch, wherein the starch is obtained from a

transformed plant, the genome of which is augmented with an expression cassette encoding the at least one enzyme; and adding the sweetened starch to a product to produce a sweetened starch containing product. The transformed plant may be selected from the group consisting of corn, soybean, rye, oats, barley, wheat, rice and sugar cane. The at least one enzyme may be hyperthermophilic. The at least one enzyme may be  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase, glucose isomerase, or any combination thereof.

A farinaceous food product and sweetened starch-containing product is provided for herein.

The invention is also directed to a method to sweeten a polysaccharide-containing fruit or vegetable comprising treating a fruit or vegetable comprising at least one polysaccharide processing enzyme under conditions which activate the at least one enzyme, thereby processing the polysaccharide in the fruit or vegetable to form sugar, yielding a sweetened fruit or vegetable, wherein the fruit or vegetable is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one polysaccharide processing enzyme. The fruit or vegetable is selected from the group consisting of potato, tomato, banana, squash, peas, and beans. The at least one enzyme may be hyperthermophilic.

The present invention is further directed to a method of preparing an aqueous solution comprising sugar comprising treating starch granules obtained from the plant part under conditions which activate the at least one enzyme, thereby yielding an aqueous solution comprising sugar.

Another embodiment is directed to a method of preparing starch derived products from grain that does not involve wet or dry milling grain prior to recovery of starch-derived products comprising treating a plant part comprising starch granules and at least one starch processing enzyme under conditions which activate the at least one enzyme thereby processing the starch granules to form an aqueous solution comprising dextrans or sugars, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one starch processing enzyme; and collecting the aqueous solution comprising the starch derived product. The at least one starch processing enzyme may be hyperthermophilic.

A method of isolating an  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\alpha$ -glucosidase, and pullulanase comprising culturing a transformed plant containing the  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\alpha$ -glucosidase, or pullulanase and isolating the  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\alpha$ -glucosidase or pullulanase therefrom is further provided. Also provided is a method of isolating a xylanase, cellulase, glucanase, beta glucosidase, protease, esterase, phytase or lipase comprising culturing a transformed plant containing the xylanase, cellulase, glucanase, beta glucosidase, protease, esterase, phytase or lipase and isolating the xylanase, cellulase, glucanase, esterase, beta glucosidase, protease, esterase, phytase or lipase .

A method of preparing maltodextrin comprising mixing transgenic grain with water, heating said mixture, separating solid from the dextrin syrup generated, and collecting the maltodextrin. The transgenic grain comprises at least one starch processing enzyme. The starch processing enzyme may be  $\alpha$ -amylase, glucoamylase,  $\alpha$ -glucosidase, and glucose isomerase. Moreover, maltodextrin produced by the method is provided as well as composition produced by this method.

A method of preparing dextrans, or sugars from grain that does not involve mechanical disruption of the grain prior to recovery of starch-derived comprising: treating a plant part comprising starch granules and at least one starch processing enzyme under conditions which activate the at least one enzyme thereby processing the starch granules to form an aqueous solution comprising dextrans or sugars, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one processing enzyme; and collecting the aqueous solution comprising sugar and/or dextrans is provided.

The present invention is further directed to a method of producing fermentable sugar comprising treating a plant part comprising starch granules and at least one starch processing enzyme under conditions which activate the at least one enzyme thereby processing the starch granules to form an aqueous solution comprising dextrans or sugars, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one processing enzyme; and



collecting the aqueous solution comprising the fermentable sugar.

Moreover, a maize plant stably transformed with a vector comprising a hyperthermophilic  $\alpha$ -amylase is provided herein. For example, preferably, a maize plant stably transformed with a vector comprising a polynucleotide sequence that encodes  $\alpha$ -amylase that is greater than 60% identical to SEQ ID NO: 1 or SEQ ID NO: 51 is encompassed.

### **Brief Description of the Figures**

Figures 1A and 1B illustrate the activity of  $\alpha$ -amylase expressed in corn kernels and in the endosperm from segregating T1 kernels from pNOV6201 plants and from six pNOV6200 lines.

Figure 2 illustrates the activity of  $\alpha$ -amylase in segregating T1 kernels from pNOV6201 lines.

Figure 3 depicts the amount of ethanol produced upon fermentation of mashes of transgenic corn containing thermostable 797GL3 alpha amylase that were subjected to liquefaction times of up to 60 minutes at 85°C and 95°C. This figure illustrates that the ethanol yield at 72 hours of fermentation was almost unchanged from 15 minutes to 60 minutes of liquefaction. Moreover, it shows that mash produced by liquefaction at 95°C produced more ethanol at each time point than mash produced by liquefaction at 85°C.

Figure 4 depicts the amount of residual starch (%) remaining after fermentation of mashes of transgenic corn containing thermostable alpha amylase that were subjected to a liquefaction time of up to 60 minutes at 85°C and 95°C. This figure illustrates that the ethanol yield at 72 hours of fermentation was almost unchanged from 15 minutes to 60 minutes of liquefaction. Moreover, it shows that mash produced by liquefaction at 95°C produced more ethanol at each time point than mash produced by liquefaction at 85°C.

Figure 5 depicts the ethanol yields for mashes of a transgenic corn, control corn, and various mixtures thereof prepared at 85°C and 95°C. This figure illustrates that the transgenic corn comprising  $\alpha$ -amylase results in significant improvement in making starch available for fermentation since there was a reduction of starch left over after fermentation.

Figure 6 depicts the amount of residual starch measured in dried stillage following fermentation for mashes of a transgenic grain, control corn, and various mixtures thereof at prepared at 85°C and 95°C.

Figure 7 depicts the ethanol yields as a function of fermentation time of a sample comprising 3% transgenic corn over a period of 20-80 hours at various pH ranges from 5.2-6.4. The figure illustrates that the fermentation conducted at a lower pH proceeds faster than at a pH of 6.0 or higher.

Figure 8 depicts the ethanol yields during fermentation of a mash comprising various weight percentages of transgenic corn from 0-12 wt% at various pH ranges from 5.2-6.4. This figure illustrates that the ethanol yield was independent of the amount of transgenic grain included in the sample.

Figure 9 shows the analysis of T2 seeds from different events transformed with pNOV 7005. High expression of pullulanase activity, compared to the non-transgenic control, can be detected in a number of events.

Figure 10A and 10B show the results of the HPLC analysis of the hydrolytic products generated by expressed pullulanase from starch in the transgenic corn flour. Incubation of the flour of pullulanase expressing corn in reaction buffer at 75 °C for 30 minutes results in production of medium chain oligosaccharides (degree of polymerization (DP) ~10-30) and short amylose chains (DP ~ 100 –200) from cornstarch. Figures 10A and 10B also show the effect of added calcium ions on the activity of the pullulanase.

Figures 11A and 11B depict the data generated from HPLC analysis of the starch hydrolysis product from two reaction mixtures. The first reaction indicated as 'Amylase' contains a mixture [1:1 (w/w)] of corn flour samples of  $\alpha$ -amylase expressing transgenic corn and non-transgenic corn A188; and the second reaction mixture 'Amylase + Pullulanase' contains a mixture [1:1 (w/w)] of corn flour samples of  $\alpha$ -amylase expressing transgenic corn and pullulanase expressing transgenic corn.

Figure 12 depicts the amount of sugar product in  $\mu\text{g}$  in 25  $\mu\text{l}$  of reaction mixture for two reaction mixtures. The first reaction indicated as 'Amylase' contains a mixture [1:1 (w/w)] of corn flour samples of  $\alpha$ -amylase expressing transgenic corn and non-transgenic corn A188; and

the second reaction mixture 'Amylase + Pullulanase' contains a mixture [1:1 (w/w)] of corn flour samples of  $\alpha$ -amylase expressing transgenic corn and pullulanase expressing transgenic corn.

Figure 13A and 13B shows the starch hydrolysis product from two sets of reaction mixtures at the end of 30 minutes incubation at 85°C and 95°C. For each set there are two reaction mixtures; the first reaction indicated as 'Amylase X Pullulanase' contains flour from transgenic corn (generated by cross pollination) expressing both the  $\alpha$ -amylase and the pullulanase, and the second reaction indicated as 'Amylase' mixture of corn flour samples of  $\alpha$ -amylase expressing transgenic corn and non-transgenic corn A188 in a ratio so as to obtain same amount of  $\alpha$ -amylase activity as is observed in the cross (Amylase X Pullulanase).

Figure 14 depicts the degradation of starch to glucose using non-transgenic corn seed (control), transgenic corn seed comprising the 797GL3  $\alpha$ -amylase, and a combination of 797GL3 transgenic corn seed with Mal A  $\alpha$ -glucosidase.

Figure 15 depicts the conversion of raw starch at room temperature or 30°C. In this figure, the reaction mixtures 1 and 2 are a combination of water and starch at room temperature and 30°C, respectively. Reaction mixtures 3 and 4 are a combination of barley  $\alpha$ -amylase and starch at room temperature and at 30°C, respectively. Reaction mixtures 5 and 6 are combinations of *Thermoanaerobacterium* glucoamylase and starch at room temperature and 30°C, respectively. Reactions mixtures 7 and 8 are combinations of barley  $\alpha$ -amylase (sigma) and *Thermoanaerobacterium* glucoamylase and starch at room temperature and 30°C, respectively. Reaction mixtures 9 and 10 are combinations of Barley alpha-amylase (sigma) control, and starch at room temperature and 30°C, respectively. The degree of polymerization (DP) of the products of the *Thermoanaerobacterium* glucoamylase is indicated.

Figure 16 depicts the production of fructose from amylase transgenic corn flour using a combination of alpha amylase, alpha glucosidase, and glucose isomerase as described in Example 19. Amylase corn flour was mixed with enzyme solutions plus water or buffer. All reactions contained 60 mg amylase flour and a total of 600 $\mu$ l of liquid and were incubated for 2 hours at 90°C.

Figure 17 depicts the peak areas of the products of reaction with 100% amylase flour from a self-processing kernel as a function of incubation time from 0-1200 minutes at 90°C.

Figure 18 depicts the peak areas of the products of reaction with 10% transgenic amylase flour from a self-processing kernel and 90% control corn flour as a function of incubation time from 0-1200 minutes at 90°C.

Figure 19 provides the results of the HPLC analysis of transgenic amylase flour incubated at 70°, 80°, 90°, or 100° C for up to 90 minutes to assess the effect of temperature on starch hydrolysis.

Figure 20 depicts ELSD peak area for samples containing 60 mg transgenic amylase flour mixed with enzyme solutions plus water or buffer under various reaction conditions. One set of reactions was buffered with 50 mM MOPS, pH 7.0 at room temperature, plus 10mM MgSO<sub>4</sub> and 1 mM CoCl<sub>2</sub>; in a second set of reactions the metal-containing buffer solution was replaced by water. All reactions were incubated for 2 hours at 90°C.

### **Detailed Description of the Invention**

In accordance with the present invention, a “self-processing” plant or plant part has incorporated therein an isolated polynucleotide encoding a processing enzyme capable of processing, e.g., modifying, starches, polysaccharides, lipids, proteins, and the like in plants, wherein the processing enzyme can be mesophilic, thermophilic or hyperthermophilic, and may be activated by grinding, addition of water, heating, or otherwise providing favorable conditions for function of the enzyme. The isolated polynucleotide encoding the processing enzyme is integrated into a plant or plant part for expression therein. Upon expression and activation of the processing enzyme, the plant or plant part of the present invention processes the substrate upon which the processing enzyme acts. Therefore, the plant or plant parts of the present invention are capable of self-processing the substrate of the enzyme upon activation of the processing enzyme contained therein in the absence of or with reduced external sources normally required for processing these substrates. As such, the transformed plants, transformed plant cells, and transformed plant parts have “built-in” processing capabilities to process desired substrates via the enzymes incorporated therein according to this invention. Preferably, the processing enzyme-encoding polynucleotide are “genetically stable,” i.e., the polynucleotide is stably maintained in the transformed plant or plant parts of the present invention and stably inherited by

progeny through successive generations.

In accordance with the present invention, methods which employ such plants and plant parts can eliminate the need to mill or otherwise physically disrupt the integrity of plant parts prior to recovery of starch-derived products. For example, the invention provides improved methods for processing corn and other grain to recover starch-derived products. The invention also provides a method which allows for the recovery of starch granules that contain levels of starch degrading enzymes, in or on the granules, that are adequate for the hydrolysis of specific bonds within the starch without the requirement for adding exogenously produced starch hydrolyzing enzymes. The invention also provides improved products from the self-processing plant or plant parts obtained by the methods of the invention.

In addition, the “self-processing” transformed plant part, e.g., grain, and transformed plant avoid major problems with existing technology, i.e., processing enzymes are typically produced by fermentation of microbes, which requires isolating the enzymes from the culture supernatants, which costs money; the isolated enzyme needs to be formulated for the particular application, and processes and machinery for adding, mixing and reacting the enzyme with its substrate must be developed. The transformed plant of the invention or a part thereof is also a source of the processing enzyme itself as well as substrates and products of that enzyme, such as sugars, amino acids, fatty acids and starch and non-starch polysaccharides. The plant of the invention may also be employed to prepare progeny plants such as hybrids and inbreds.

### **Processing Enzymes And Polynucleotides Encoding Them**

A polynucleotide encoding a processing enzyme (mesophilic, thermophilic, or hyperthermophilic) is introduced into a plant or plant part. The processing enzyme is selected based on the desired substrate upon which it acts as found in plants or transgenic plants and/or the desired end product. For example, the processing enzyme may be a starch-processing enzyme, such as a starch-degrading or starch-isomerizing enzyme, or a non-starch processing enzyme. Suitable processing enzymes include, but are not limited to, starch degrading or isomerizing enzymes including, for example,  $\alpha$ -amylase, endo or exo-1,4, or 1,6- $\alpha$ -D, glucoamylase, glucose isomerase,  $\beta$ -amylases,  $\alpha$ -glucosidases, and other exo-amylases; and



starch debranching enzymes, such as isoamylase, pullulanase, neo-pullulanase, iso-pullulanase, amylopullulanase and the like, glycosyl transferases such as cyclodextrin glycosyltransferase and the like, cellulases such as exo-1,4- $\beta$ -cellobiohydrolase, exo-1,3- $\beta$ -D-glucanase, hemicellulase,  $\beta$ -glucosidase and the like; endoglucanases such as endo-1,3- $\beta$ -glucanase and endo-1,4- $\beta$ -glucanase and the like; L-arabinases, such as endo-1,5- $\alpha$ -L-arabinase,  $\alpha$ -arabinosidases and the like; galactanases such as endo-1,4- $\beta$ -D-galactanase, endo-1,3- $\beta$ -D-galactanase,  $\beta$ -galactosidase,  $\alpha$ -galactosidase and the like; mannanases, such as endo-1,4- $\beta$ -D-mannanase,  $\beta$ -mannosidase,  $\alpha$ -mannosidase and the like; xylanases, such as endo-1,4- $\beta$ -xylanase,  $\beta$ -D-xylosidase, 1,3- $\beta$ -D-xylanase, and the like; and pectinases; and non-starch processing enzymes, including protease, glucanase, xylanase, thioredoxin/thioredoxin reductase, esterase, phytase, and lipase.

In one embodiment, the processing enzyme is a starch-degrading enzyme selected from the group of  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucoamylase, amylopullulanase, glucose isomerase, or combinations thereof. According to this embodiment, the starch-degrading enzyme is able to allow the self-processing plant or plant part to degrade starch upon activation of the enzyme contained in the plant or plant part, as will be further described herein. The starch-degrading enzyme(s) is selected based on the desired end-products. For example, a glucose-isomerase may be selected to convert the glucose (hexose) into fructose. Alternatively, the enzyme may be selected based on the desired starch-derived end product with various chain lengths based on, e.g., a function of the extent of processing or with various branching patterns desired. For example, an  $\alpha$ -amylase, glucoamylase, or amylopullulanase can be used under short incubation times to produce dextrin products and under longer incubation times to produce shorter chain products or sugars. A pullulanase can be used to specifically hydrolyze branch points in the starch yielding a high-amylose starch, or a neopullulanase can be used to produce starch with stretches of  $\alpha$  1,4 linkages with interspersed  $\alpha$  1,6 linkages. Glucosidases could be used to produce limit dextrans, or a combination of different enzymes to make other starch derivatives.

In another embodiment, the processing enzyme is a non-starch processing enzyme selected from protease, glucanase, xylanase, phytase, lipase, cellulase, beta glucosidase and esterase. These non-starch degrading enzymes allow the self-processing plant or plant part of the

present invention to incorporate in a targeted area of the plant and, upon activation, disrupt the plant while leaving the starch granule therein intact. For example, in a preferred embodiment, the non-starch degrading enzymes target the endosperm matrix of the plant cell and, upon activation, disrupt the endosperm matrix while leaving the starch granule therein intact and more readily recoverable from the resulting material.

Combinations of processing enzymes are further envisioned by the present invention. For example, starch-processing and non-starch processing enzymes may be used in combination. Combinations of processing enzymes may be obtained by employing the use of multiple gene constructs encoding each of the enzymes. Alternatively, the individual transgenic plants stably transformed with the enzymes may be crossed by known methods to obtain a plant containing both enzymes. Another method includes the use of exogenous enzyme(s) with the transgenic plant.

The processing enzymes may be isolated or derived from any source and the polynucleotides corresponding thereto may be ascertained by one having skill in the art. For example, the processing enzyme, such as  $\alpha$ -amylase, is derived from the *Pyrococcus* (e.g., *Pyrococcus furiosus*), *Thermus*, *Thermococcus* (e.g., *Thermococcus hydrothermalis*), *Sulfolobus* (e.g., *Sulfolobus solfataricus*) *Thermotoga* (e.g., *Thermotoga maritima* and *Thermotoga neapolitana*), *Thermoanaerobacterium* (e.g. *Thermoanaerobacter tengcongensis*), *Aspergillus* (e.g., *Aspergillus shirousami* and *Aspergillus niger*), *Rhizopus* (eg., *Rhizopus oryzae*), *Thermoproteales*, *Desulfurococcus* (e.g. *Desulfurococcus amylolyticus*), *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, *Methanopyrus kandleri*, *Thermosynechococcus elongatus*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, *Aeropyrum pernix* and plants such as corn, barley, and rice.

The processing enzymes of the present invention are capable of being activated after being introduced and expressed in the genome of a plant. Conditions for activating the enzyme are determined for each individual enzyme and may include varying conditions such as temperature, pH, hydration, presence of metals, activating compounds, inactivating compounds, etc. For example, temperature-dependent enzymes may include mesophilic, thermophilic, and hyperthermophilic enzymes. Mesophilic enzymes typically have maximal activity at

temperatures between 20°- 65°C and are inactivated at temperatures greater than 70° C.

Mesophilic enzymes have significant activity at 30 to 37°C, the activity at 30 °C is preferably at least 10% of maximal activity, more preferably at least 20% of maximal activity.

Thermophilic enzymes have a maximal activity at temperatures of between 50 and 80° C and are inactivated at temperatures greater than 80°C . A thermophilic enzyme will preferably have less than 20% of maximal activity at 30°C, more preferably less than 10% of maximal activity.

A "hyperthermophilic" enzyme has activity at even higher temperatures.

Hyperthermophilic enzymes have a maximal activity at temperatures greater than 80° C and retain activity at temperatures at least 80°C, more preferably retain activity at temperatures of at least 90°C and most preferably retain activity at temperatures of at least 95°C.

Hyperthermophilic enzymes also have reduced activity at low temperatures. A hyperthermophilic enzyme may have activity at 30°C that is less than 10% of maximal activity, and preferably less than 5% of maximal activity.

The polynucleotide encoding the processing enzyme is preferably modified to include codons that are optimized for expression in a selected organism such as a plant (see, e.g., Wada et al., Nucl. Acids Res., 18:2367 (1990), Murray et al., Nucl. Acids Res., 17:477 (1989), U.S. Patent Nos. 5,096,825, 5,625,136, 5,670,356 and 5,874,304). Codon optimized sequences are synthetic sequences, i.e., they do not occur in nature, and preferably encode the identical polypeptide (or an enzymatically active fragment of a full length polypeptide which has substantially the same activity as the full length polypeptide) encoded by the non-codon optimized parent polynucleotide which encodes a processing enzyme. It is preferred that the polypeptide is biochemically distinct or improved, e.g., via recursive mutagenesis of DNA encoding a particular processing enzyme, from the parent source polypeptide such that its performance in the process application is improved. Preferred polynucleotides are optimized for expression in a target host plant and encode a processing enzyme. Methods to prepare these enzymes include mutagenesis, e.g., recursive mutagenesis and selection. Methods for mutagenesis and nucleotide sequence alterations are well-known in the art. See, for example, Kunkel, Proc. Natl. Acad. Sci. USA, 82:488, (1985); Kunkel et al., Methods in Enzymol., 154:367 (1987); US

Patent No. 4,873,192; Walker and Gaastra, eds. (1983) Techniques in Molecular Biology (MacMillan Publishing Company, New York) and the references cited therein and Arnold et al., Chem. Eng. Sci., 51:5091 (1996)). Methods to optimize the expression of a nucleic acid segment in a target plant or organism are well-known in the art. Briefly, a codon usage table indicating the optimal codons used by the target organism is obtained and optimal codons are selected to replace those in the target polynucleotide and the optimized sequence is then chemically synthesized. Preferred codons for maize are described in U.S. Patent No. 5,625,136.

Complementary nucleic acids of the polynucleotides of the present invention are further envisioned. An example of low stringency conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60°C to 65°C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C.

Moreover, polynucleotides encoding an “enzymatically active” fragment of the processing enzymes are further envisioned. As used herein, “enzymatically active” means a polypeptide fragment of the processing enzyme that has substantially the same biological activity as the processing enzyme to modify the substrate upon which the processing enzyme normally acts under appropriate conditions.

In a preferred embodiment, the polynucleotide of the present invention is a maize-optimized polynucleotide encoding  $\alpha$ -amylase, such as provided in SEQ ID NOs:2, 9, 46, and 52. In another preferred embodiment, the polynucleotide is a maize-optimized polynucleotide encoding pullulanase, such as provided in SEQ ID NOs: 4 and 25. In yet another preferred embodiment, the polynucleotide is a maize-optimized polynucleotide encoding  $\alpha$ -glucosidase as provided in SEQ ID NO:6. Another preferred polynucleotide is the maize-optimized polynucleotide encoding glucose isomerase having SEQ ID NO: 19, 21, 37, 39, 41, or 43. In

another embodiment, the maize-optimized polynucleotide encoding glucoamylase as set forth in SEQ ID NO: 46, 48, or 50 is preferred. Moreover, a maize-optimized polynucleotide for glucanase/mannanase fusion polypeptide is provided in SEQ ID NO: 57. The invention further provides for complements of such polynucleotides, which hybridize under moderate, or preferably under low stringency, hybridization conditions and which encodes a polypeptide having  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucose isomerase, glucoamylase, glucanase, or mannanase activity, as the case may be.

The polynucleotide may be used interchangeably with "nucleic acid" or "polynucleic acid" and refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, composed of monomers (nucleotides) containing a sugar, phosphate and a base, which is either a purine or pyrimidine. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides, which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues.

"Variants" or substantially similar sequences are further encompassed herein. For nucleotide sequences, variants include those sequences that, because of the degeneracy of the genetic code, encode the identical amino acid sequence of the native protein. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR), hybridization techniques, and ligation reassembly techniques. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis, which encode the native protein, as well as those that encode a polypeptide having amino acid substitutions. Generally, nucleotide sequence variants of the invention will have at least 40%, 50%, 60%, preferably 70%, more preferably 80%, even more preferably 90%,



most preferably 99%, and single unit percentage identity to the native nucleotide sequence based on these classes. For example, 71%, 72%, 73% and the like, up to at least the 90% class. Variants may also include a full-length gene corresponding to an identified gene fragment.

#### **Regulatory Sequences: Promoters/Signal Sequences/Selectable Markers**

The polynucleotide sequences encoding the processing enzyme of the present invention may be operably linked to polynucleotide sequences encoding localization signals or signal sequence (at the N- or C-terminus of a polypeptide), e.g., to target the hyperthermophilic enzyme to a particular compartment within a plant. Examples of such targets include, but are not limited to, the vacuole, endoplasmic reticulum, chloroplast, amyloplast, starch granule, or cell wall, or to a particular tissue, e.g., seed. The expression of a polynucleotide encoding a processing enzyme having a signal sequence in a plant, in particular, in conjunction with the use of a tissue-specific or inducible promoter, can yield high levels of localized processing enzyme in the plant. Numerous signal sequences are known to influence the expression or targeting of a polynucleotide to a particular compartment or outside a particular compartment. Suitable signal sequences and targeting promoters are known in the art and include, but are not limited to, those provided herein.

For example, where expression in specific tissues or organs is desired, tissue-specific promoters may be used. In contrast, where gene expression in response to a stimulus is desired, inducible promoters are the regulatory elements of choice. Where continuous expression is desired throughout the cells of a plant, constitutive promoters are utilized. Additional regulatory sequences upstream and/or downstream from the core promoter sequence may be included in expression constructs of transformation vectors to bring about varying levels of expression of heterologous nucleotide sequences in a transgenic plant.

A number of plant promoters have been described with various expression characteristics. Examples of some constitutive promoters which have been described include the rice actin 1 (Wang et al., Mol. Cell. Biol., 12:3399 (1992); U.S. Patent No. 5,641,876), CaMV 35S (Odell et al., Nature, 313:810 (1985)), CaMV 19S (Lawton et al., 1987), nos (Ebert et al., 1987), Adh (Walker et al., 1987), sucrose synthase (Yang & Russell, 1990), and the ubiquitin promoters.

Vectors for use in tissue-specific targeting of genes in transgenic plants will typically include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure. These include, for example, the *rbcS* promoter, specific for green tissue; the *ocs*, *nos* and *mas* promoters which have higher activity in roots or wounded leaf tissue; a truncated (-90 to +8) 35S promoter which directs enhanced expression in roots, an  $\alpha$ -tubulin gene that directs expression in roots and promoters derived from zein storage protein genes which direct expression in endosperm.

Tissue specific expression may be functionally accomplished by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired. For example, a gene coding for a lipase may be introduced such that it is expressed in all tissues using the 35S promoter from Cauliflower Mosaic Virus. Expression of an antisense transcript of the lipase gene in a maize kernel, using for example a zein promoter, would prevent accumulation of the lipase protein in seed. Hence the protein encoded by the introduced gene would be present in all tissues except the kernel.

Moreover, several tissue-specific regulated genes and/or promoters have been reported in plants. Some reported tissue-specific genes include the genes encoding the seed storage proteins (such as napin, cruciferin, beta-conglycinin, and phaseolin) zein or oil body proteins (such as oleosin), or genes involved in fatty acid biosynthesis (including acyl carrier protein, stearyl-ACP desaturase, and fatty acid desaturases (*fad 2-1*)), and other genes expressed during embryo development (such as *Bce4*, see, for example, EP 255378 and Kridl et al., Seed Science Research, 1:209 (1991)). Examples of tissue-specific promoters, which have been described include the lectin (Vodkin, Prog. Clin. Biol. Res., 138:87 (1983); Lindstrom et al., Dev. Genet., 11:160 (1990)), corn alcohol dehydrogenase 1 (Vogel et al., 1989; Dennis et al., Nucleic Acids Res., 12:3983 (1984)), corn light harvesting complex (Simpson, 1986; Bansal et al., Proc. Natl. Acad. Sci. USA, 89:3654 (1992)), corn heat shock protein (Odell et al., 1985; Rochester et al., 1986), pea small subunit RuBP carboxylase (Poulsen et al., 1986; Cashmore et al., 1983), Ti

plasmid mannopine synthase (Langridge et al., 1989), Ti plasmid nopaline synthase (Langridge et al., 1989), petunia chalcone isomerase (vanTunen et al., EMBO J., 7:1257(1988)), bean glycine rich protein 1 (Keller et al., Genes Dev., 3:1639 (1989)), truncated CaMV 35s (Odell et al., Nature, 313:810 (1985)), potato patatin (Wenzler et al., Plant Mol. Biol., 13:347 (1989)), root cell (Yamamoto et al., Nucleic Acids Res., 18:7449 (1990)), maize zein (Reina et al., Nucleic Acids Res., 18:6425 (1990); Kriz et al., Mol. Gen. Genet., 207:90 (1987); Wandelt et al., Nucleic Acids Res., 17:2354 (1989); Langridge et al., Cell, 34:1015 (1983); Reina et al., Nucleic Acids Res., 18:7449 (1990)), globulin-1 (Belanger et al., Genetics, 129:863 (1991)),  $\alpha$ -tubulin, cab (Sullivan et al., Mol. Gen. Genet., 215:431 (1989)), PEPCase (Hudspeth & Grula, 1989), R gene complex-associated promoters (Chandler et al., Plant Cell, 1:1175 (1989)), and chalcone synthase promoters (Franken et al., EMBO J., 10:2605 (1991)). Particularly useful for seed-specific expression is the pea vicilin promoter (Czako et al., Mol. Gen. Genet., 235:33 (1992)). (See also U.S. Pat. No. 5,625,136, herein incorporated by reference.) Other useful promoters for expression in mature leaves are those that are switched on at the onset of senescence, such as the SAG promoter from Arabidopsis (Gan et al., Science, 270:1986 (1995)).

A class of fruit-specific promoters expressed at or during anthesis through fruit development, at least until the beginning of ripening, is discussed in U.S. 4,943,674, the disclosure of which is hereby incorporated by reference. cDNA clones that are preferentially expressed in cotton fiber have been isolated (John et al., Proc. Natl. Acad. Sci. USA, 89:5769 (1992)). cDNA clones from tomato displaying differential expression during fruit development have been isolated and characterized (Mansson et al., Gen. Genet., 200:356 (1985), Slater et al., Plant Mol. Biol., 5:137 (1985)). The promoter for polygalacturonase gene is active in fruit ripening. The polygalacturonase gene is described in U.S. Patent No. 4,535,060, U.S. Patent No. 4,769,061, U.S. Patent No. 4,801,590, and U.S. Patent No. 5,107,065, which disclosures are incorporated herein by reference.

Other examples of tissue-specific promoters include those that direct expression in leaf cells following damage to the leaf (for example, from chewing insects), in tubers (for example, patatin gene promoter), and in fiber cells (an example of a developmentally-regulated fiber cell

protein is E6 (John et al., Proc. Natl. Acad. Sci. USA, 89:5769 (1992)). The E6 gene is most active in fiber, although low levels of transcripts are found in leaf, ovule and flower.

The tissue-specificity of some "tissue-specific" promoters may not be absolute and may be tested by one skilled in the art using the diphtheria toxin sequence. One can also achieve tissue-specific expression with "leaky" expression by a combination of different tissue-specific promoters (Beals et al., Plant Cell, 9:1527 (1997)). Other tissue-specific promoters can be isolated by one skilled in the art (see U.S. 5,589,379).

In one embodiment, the direction of the product from a polysaccharide hydrolysis gene, such as  $\alpha$ -amylase, may be targeted to a particular organelle such as the apoplast rather than to the cytoplasm. This is exemplified by the use of the maize  $\gamma$ -zein N-terminal signal sequence (SEQ ID NO:17), which confers apoplast-specific targeting of proteins. Directing the protein or enzyme to a specific compartment will allow the enzyme to be localized in a manner that it will not come into contact with the substrate. In this manner the enzymatic action of the enzyme will not occur until the enzyme contacts its substrate. The enzyme can be contacted with its substrate by the process of milling (physical disruption of the cell integrity), or heating the cells or plant tissues to disrupt the physical integrity of the plant cells or organs that contain the enzyme. For example a mesophilic starch-hydrolyzing enzyme can be targeted to the apoplast or to the endoplasmic reticulum and so as not to come into contact with starch granules in the amyloplast. Milling of the grain will disrupt the integrity of the grain and the starch hydrolyzing enzyme will then contact the starch granules. In this manner the potential negative effects of co-localization of an enzyme and its substrate can be circumvented.

In another embodiment, a tissue-specific promoter includes the endosperm-specific promoters such as the maize  $\gamma$ -zein promoter (exemplified by SEQ ID NO:12) or the maize ADP-gpp promoter (exemplified by SEQ ID NO:11, which includes a 5' untranslated and an intron sequence) or a Q protein promoter (exemplified by SEQ ID NO: 98) or a rice glutelin 1 promoter (exemplified in SEQ ID NO:67). Thus, the present invention includes an isolated polynucleotide comprising a promoter comprising SEQ ID NO: 11, 12, 67, or 98, a polynucleotide which hybridizes to the complement thereof under low stringency hybridization

conditions, or a fragment thereof which has promoter activity, e.g., at least 10%, and preferably at least 50%, the activity of a promoter having SEQ ID NO:11, 12, 67, or 98.

In another embodiment of the invention, the polynucleotide encodes a hyperthermophilic processing enzyme that is operably linked to a chloroplast (amyloplast) transit peptide (CTP) and a starch binding domain, e.g., from the *waxy* gene. An exemplary polynucleotide in this embodiment encodes SEQ ID NO:10 ( $\alpha$ -amylase linked to the starch binding domain from *waxy*). Other exemplary polynucleotides encode a hyperthermophilic processing enzyme linked to a signal sequence that targets the enzyme to the endoplasmic reticulum and secretion to the apoplast (exemplified by a polynucleotide encoding SEQ ID NO:13, 27, or 30, which comprises the N-terminal sequence from maize  $\gamma$ -zein operably linked to  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucose isomerase, respectively), a hyperthermophilic processing enzyme linked to a signal sequence which retains the enzyme in the endoplasmic reticulum (exemplified by a polynucleotide encoding SEQ ID NO:14, 26, 28, 29, 33, 34, 35, or 36, which comprises the N-terminal sequence from maize  $\gamma$ -zein operably linked to the hyperthermophilic enzyme, which is operably linked to SEKDEL, wherein the enzyme is  $\alpha$ -amylase, malA  $\alpha$ -glucosidase, *T. maritima* glucose isomerase, *T. neapolitana* glucose isomerase), a hyperthermophilic processing enzyme linked to an N-terminal sequence that targets the enzyme to the amyloplast (exemplified by a polynucleotide encoding SEQ ID NO:15, which comprises the N-terminal amyloplast targeting sequence from *waxy* operably linked to  $\alpha$ -amylase), a hyperthermophilic fusion polypeptide which targets the enzyme to starch granules (exemplified by a polynucleotide encoding SEQ ID NO:16, which comprises the N-terminal amyloplast targeting sequence from *waxy* operably linked to an  $\alpha$ -amylase/*waxy* fusion polypeptide comprising the *waxy* starch binding domain), a hyperthermophilic processing enzyme linked to an ER retention signal (exemplified by a polynucleotide encoding SEQ ID NO:38 and 39). Moreover, a hyperthermophilic processing enzyme may be linked to a raw-starch binding site having the amino acid sequence (SEQ ID NO:53), wherein the polynucleotide encoding the processing enzyme is linked to the maize-optimized nucleic acid sequence (SEQ ID NO:54) encoding this binding site.

Several inducible promoters have been reported. Many are described in a review by Gatz, in Current Opinion in Biotechnology, 7:168 (1996) and Gatz, C., Annu. Rev. Plant Physiol.



Plant Mol. Biol., 48:89 (1997). Examples include tetracycline repressor system, Lac repressor system, copper-inducible systems, salicylate-inducible systems (such as the PR1a system), glucocorticoid-inducible (Aoyama T. et al., N-H Plant Journal, 11:605 (1997)) and ecdysone-inducible systems. Other inducible promoters include ABA- and turgor-inducible promoters, the promoter of the auxin-binding protein gene (Schwob et al., Plant J., 4:423 (1993)), the UDP glucose flavonoid glycosyl-transferase gene promoter (Ralston et al., Genetics, 119:185 (1988)), the MPI proteinase inhibitor promoter (Cordero et al., Plant J., 6:141 (1994)), and the glyceraldehyde-3-phosphate dehydrogenase gene promoter (Kohler et al., Plant Mol. Biol., 29:1293 (1995); Quigley et al., J. Mol. Evol., 29:412 (1989); Martinez et al., J. Mol. Biol., 208:551 (1989)). Also included are the benzene sulphonamide-inducible (U.S. 5364,780) and alcohol-inducible (WO 97/06269 and WO 97/06268) systems and glutathione S-transferase promoters.

Other studies have focused on genes inducibly regulated in response to environmental stress or stimuli such as increased salinity, drought, pathogen and wounding. (Graham et al., J. Biol. Chem., 260:6555 (1985); Graham et al., J. Biol. Chem., 260:6561 (1985), Smith et al., Planta, 168:94 (1986)). Accumulation of metallocarboxypeptidase-inhibitor protein has been reported in leaves of wounded potato plants (Graham et al., Biochem. Biophys. Res. Comm., 101:1164 (1981)). Other plant genes have been reported to be induced by methyl jasmonate, elicitors, heat-shock, anaerobic stress, or herbicide safeners.

Regulated expression of a chimeric transacting viral replication protein can be further regulated by other genetic strategies, such as, for example, Cre-mediated gene activation (Odell et al. Mol. Gen. Genet., 113:369 (1990)). Thus, a DNA fragment containing 3' regulatory sequence bound by lox sites between the promoter and the replication protein coding sequence that blocks the expression of a chimeric replication gene from the promoter can be removed by Cre-mediated excision and result in the expression of the trans-acting replication gene. In this case, the chimeric Cre gene, the chimeric trans-acting replication gene, or both can be under the control of tissue- and developmental-specific or inducible promoters. An alternate genetic strategy is the use of tRNA suppressor gene. For example, the regulated expression of a tRNA suppressor gene can conditionally control expression of a trans-acting replication protein coding sequence containing an appropriate termination codon (Ulmasov et al. Plant Mol. Biol., 35:417

(1997)). Again, either the chimeric tRNA suppressor gene, the chimeric transacting replication gene, or both can be under the control of tissue- and developmental-specific or inducible promoters.

Preferably, in the case of a multicellular organism, the promoter can also be specific to a particular tissue, organ or stage of development. Examples of such promoters include, but are not limited to, the *Zea mays* ADP-gpp and the *Zea mays*  $\gamma$ -zein promoter and the *Zea mays* globulin promoter .

Expression of a gene in a transgenic plant may be desired only in a certain time period during the development of the plant. Developmental timing is frequently correlated with tissue specific gene expression. For example, expression of zein storage proteins is initiated in the endosperm about 15 days after pollination.

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This will generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, e.g., vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane.

A signal sequence such as the maize  $\gamma$ -zein N-terminal signal sequence for targeting to the endoplasmic reticulum and secretion into the apoplast may be operably linked to a polynucleotide encoding a hyperthermophilic processing enzyme in accordance with the present invention (Torrent et al., 1997). For example, SEQ ID NOs:13, 27, and 30 provides for a polynucleotide encoding a hyperthermophilic enzyme operably linked to the N-terminal sequence from maize  $\gamma$ -zein protein. Another signal sequence is the amino acid sequence SEKDEL for retaining polypeptides in the endoplasmic reticulum (Munro and Pelham, 1987). For example, a polynucleotide encoding SEQ ID NOS:14, 26, 28, 29, 33, 34, 35, or 36, which comprises the N-terminal sequence from maize  $\gamma$ -zein operably linked to a processing enzyme

which is operably linked to SEKDEL. A polypeptide may also be targeted to the amyloplast by fusion to the waxy amyloplast targeting peptide (Klosgen et al., 1986) or to a starch granule. For example, the polynucleotide encoding a hyperthermophilic processing enzyme may be operably linked to a chloroplast (amyloplast) transit peptide (CTP) and a starch binding domain, e.g., from the *waxy* gene. SEQ ID NO:10 exemplifies  $\alpha$ -amylase linked to the starch binding domain from *waxy*. SEQ ID NO:15 exemplifies the N-terminal sequence amyloplast targeting sequence from *waxy* operably linked to  $\alpha$ -amylase. Moreover, the polynucleotide encoding the processing enzyme may be fused to target starch granules using the waxy starch binding domain. For example, SEQ ID NO:16 exemplifies a fusion polypeptide comprising the N-terminal amyloplast targeting sequence from *waxy* operably linked to an  $\alpha$ -amylase/*waxy* fusion polypeptide comprising the waxy starch binding domain.

The polynucleotides of the present invention, in addition to processing signals, may further include other regulatory sequences, as is known in the art. "Regulatory sequences" and "suitable regulatory sequences" each refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences include enhancers, promoters, translation leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences, which may be a combination of synthetic and natural sequences.

Selectable markers may also be used in the present invention to allow for the selection of transformed plants and plant tissue, as is well-known in the art. One may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can select for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by screening (e.g., the R-locus trait). Of course,

many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA; small active enzymes detectable in extracellular solution (e.g.,  $\alpha$ -amylase,  $\beta$ -lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (e.g., proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). For example, the maize HPRG (Steifel et al., The Plant Cell, 2:785 (1990)) molecule is well characterized in terms of molecular biology, expression and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller et al., EMBO Journal, 8:1309 (1989)) could be modified by the addition of an antigenic site to create a screenable marker.

#### a. Selectable Markers

Possible selectable markers for use in connection with the present invention include, but are not limited to, a neo or nptII gene (Potrykus et al., Mol. Gen. Genet., 199:183 (1985)) which codes for kanamycin resistance and can be selected for using kanamycin, G418, and the like; a bar gene which confers resistance to the herbicide phosphinothricin; a gene which encodes an

altered EPSP synthase protein (Hinchee et al., Biotech., 6:915 (1988)) thus conferring glyphosate resistance; a nitrilase gene such as bxn from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker et al., Science, 242:419 (1988)); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonyleurea or other ALS-inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate-resistant DHFR gene (Thillet et al., J. Biol. Chem., 263:12500 (1988)); a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; a phosphomannose isomerase (PMI) gene; a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan; the hph gene which confers resistance to the antibiotic hygromycin; or the mannose-6-phosphate isomerase gene (also referred to herein as the phosphomannose isomerase gene), which provides the ability to metabolize mannose (U.S. Patent Nos. 5,767,378 and 5,994,629). One skilled in the art is capable of selecting a suitable selectable marker gene for use in the present invention. Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (European Patent Application 0,218,571, 1987).

An illustrative embodiment of a selectable marker gene capable of being used in systems to select transformants are the genes that encode the enzyme phosphinothricin acetyltransferase, such as the bar gene from *Streptomyces hygroscopicus* or the pat gene from *Streptomyces viridochromogenes*. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami et al., Mol. Gen. Genet., 205:42 (1986); Twell et al., Plant Physiol., 91:1270 (1989)) causing rapid accumulation of ammonia and cell death. The success in using this selective system in conjunction with monocots was particularly surprising because of the major difficulties which have been reported in transformation of cereals (Potrykus, Trends Biotech., 7:269 (1989)).

Where one desires to employ a bialaphos resistance gene in the practice of the invention, a particularly useful gene for this purpose is the bar or pat genes obtainable from species of *Streptomyces* (e.g., ATCC No. 21,705). The cloning of the bar gene has been described (Murakami et al., Mol. Gen. Genet., 205:42 (1986); Thompson et al., EMBO Journal, 6:2519 (1987)) as has the use of the bar gene in the context of plants other than monocots (De Block et al., EMBO Journal, 6:2513 (1987); De Block et al., Plant Physiol., 91:694 (1989)).



### b. Screenable Markers

Screenable markers that may be employed include, but are not limited to, a  $\beta$ -glucuronidase or uidA gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta et al., in Chromosome Structure and Function, pp. 263-282 (1988)); a  $\beta$ -lactamase gene (Sutcliffe, PNAS USA, 75:3737 (1978)), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a xyleE gene (Zukowsky et al., PNAS USA, 80:1101 (1983)) which encodes a catechol dioxygenase that can convert chromogenic catechols; an  $\alpha$ -amylase gene (Ikuta et al., Biotech., 8:241 (1990)); a tyrosinase gene (Katz et al., J. Gen. Microbiol., 129:2703 (1983)) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily detectable compound melanin; a  $\beta$ -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (lux) gene (Ow et al., Science, 234:856 (1986)), which allows for bioluminescence detection; or an aequorin gene (Prasher et al., Biochem. Biophys. Res. Comm., 126:1259 (1985)), which may be employed in calcium-sensitive bioluminescence detection, or a green fluorescent protein gene (Niedz et al., Plant Cell Reports, 14: 403 (1995)).

Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. A gene from the R gene complex is suitable for maize transformation, because the expression of this gene in transformed cells does not harm the cells. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR112, a K55 derivative which is r-g, b, P1. Alternatively any genotype of maize can be utilized if the C1 and R alleles are introduced together. A further screenable marker contemplated for use in the

present invention is firefly luciferase, encoded by the lux gene. The presence of the lux gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It is also envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening.

The polynucleotides used to transform the plant may include, but is not limited to, DNA from plant genes and non-plant genes such as those from bacteria, yeasts, animals or viruses. The introduced DNA can include modified genes, portions of genes, or chimeric genes, including genes from the same or different maize genotype. The term "chimeric gene" or "chimeric DNA" is defined as a gene or DNA sequence or segment comprising at least two DNA sequences or segments from species which do not combine DNA under natural conditions, or which DNA sequences or segments are positioned or linked in a manner which does not normally occur in the native genome of the untransformed plant.

Expression cassettes comprising the polynucleotide encoding a hyperthermophilic processing enzyme, and preferably a codon-optimized polynucleotide is further provided. It is preferred that the polynucleotide in the expression cassette (the first polynucleotide) is operably linked to regulatory sequences, such as a promoter, an enhancer, an intron, a termination sequence, or any combination thereof, and, optionally, to a second polynucleotide encoding a signal sequence (N- or C-terminal) which directs the enzyme encoded by the first polynucleotide to a particular cellular or subcellular location. Thus, a promoter and one or more signal sequences can provide for high levels of expression of the enzyme in particular locations in a plant, plant tissue or plant cell. Promoters can be constitutive promoters, inducible (conditional) promoters or tissue-specific promoters, e.g., endosperm-specific promoters such as the maize  $\gamma$ -zein promoter (exemplified by SEQ ID NO:12) or the maize ADP-gpp promoter (exemplified by SEQ ID NO:11, which includes a 5' untranslated and an intron sequence). The invention also provides an isolated polynucleotide comprising a promoter comprising SEQ ID NO:11 or 12, a polynucleotide which hybridizes to the complement thereof under low stringency hybridization conditions, or a fragment thereof which has promoter activity, e.g., at least 10%, and preferably at least 50%, the activity of a promoter having SEQ ID NO:11 or 12. Also provided are vectors which comprise the expression cassette or polynucleotide of the invention and transformed cells

comprising the polynucleotide, expression cassette or vector of the invention. A vector of the invention can comprise a polynucleotide sequence which encodes more than one hyperthermophilic processing enzyme of the invention, which sequence can be in sense or antisense orientation, and a transformed cell may comprise one or more vectors of the invention. Preferred vectors are those useful to introduce nucleic acids into plant cells.

### Transformation

The expression cassette, or a vector construct containing the expression cassette may be inserted into a cell. The expression cassette or vector construct may be carried episomally or integrated into the genome of the cell. The transformed cell may then be grown into a transgenic plant. Accordingly, the invention provides the products of the transgenic plant. Such products may include, but are not limited to, the seeds, fruit, progeny, and products of the progeny of the transgenic plant.

A variety of techniques are available and known to those skilled in the art for introduction of constructs into a cellular host. Transformation of bacteria and many eukaryotic cells may be accomplished through use of polyethylene glycol, calcium chloride, viral infection, phage infection, electroporation and other methods known in the art. Techniques for transforming plant cells or tissue include transformation with DNA employing *A. tumefaciens* or *A. rhizogenes* as the transforming agent, electroporation, DNA injection, microprojectile bombardment, particle acceleration, etc. (See, for example, EP 295959 and EP 138341).

In one embodiment, binary type vectors of Ti and Ri plasmids of *Agrobacterium* spp. Ti-derived vectors are used to transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton, rape, tobacco, and rice (Pacciotti et al. Bio/Technology, 3:241 (1985); Byrne et al. Plant Cell Tissue and Organ Culture, 8:3 (1987); Sukhapinda et al. Plant Mol. Biol., 8:209 (1987); Lorz et al. Mol. Gen. Genet., 199:178 (1985); Potrykus Mol. Gen. Genet., 199:183 (1985); Park et al., J. Plant Biol., 38:365 (1985); Hiei et al., Plant J., 6:271(1994)). The use of T-DNA to transform plant cells has received extensive study and is amply described (EP 120516; Hoekema, In: The Binary Plant Vector System. Offset-drukkerij Kanters B.V.; Alblasterdam (1985), Chapter V; Knauf, et al., Genetic Analysis of Host Range Expression by *Agrobacterium* In: Molecular Genetics of the

Bacteria-Plant Interaction, Puhler, A. ed., Springer-Verlag, New York, 1983, p. 245; and An. et al., EMBO J., 4:277 (1985)).

Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs (see EP 295959), techniques of electroporation (Fromm et al. Nature (London), 319:791 (1986), or high velocity ballistic bombardment with metal particles coated with the nucleic acid constructs (Kline et al. Nature (London) 327:70 (1987), and U.S. Patent No. 4,945,050). Once transformed, the cells can be regenerated by those skilled in the art. Of particular relevance are the recently described methods to transform foreign genes into commercially important crops, such as rapeseed (De Block et al., Plant Physiol. 91:694-701 (1989)), sunflower (Everett et al., Bio/Technology, 5:1201(1987)), soybean (McCabe et al., Bio/Technology, 6:923 (1988); Hinchey et al., Bio/Technology, 6:915 (1988); Chee et al., Plant Physiol., 91:1212 (1989); Christou et al., Proc. Natl. Acad. Sci USA, 86:7500 (1989) EP 301749), rice (Hiei et al., Plant J., 6:271 (1994)), and corn (Gordon Kamm et al., Plant Cell, 2:603 (1990); Fromm et al., Biotechnology, 8:833, (1990)).

Expression vectors containing genomic or synthetic fragments can be introduced into protoplasts or into intact tissues or isolated cells. Preferably expression vectors are introduced into intact tissue. General methods of culturing plant tissues are provided, for example, by Maki et al. "Procedures for Introducing Foreign DNA into Plants" in Methods in Plant Molecular Biology & Biotechnology, Glich et al. (Eds.), pp. 67-88 CRC Press (1993); and by Phillips et al. "Cell-Tissue Culture and In-Vitro Manipulation" in Corn & Corn Improvement, 3rd Edition 10, Sprague et al. (Eds.) pp. 345-387, American Society of Agronomy Inc. (1988).

In one embodiment, expression vectors may be introduced into maize or other plant tissues using a direct gene transfer method such as microprojectile-mediated delivery, DNA injection, electroporation and the like. Expression vectors are introduced into plant tissues using the microprojectile media delivery with the biolistic device. See, for example, Tomes et al. "Direct DNA transfer into intact plant cells via microprojectile bombardment" in Gamborg and Phillips (Eds.) Plant Cell, Tissue and Organ Culture: Fundamental Methods, Springer Verlag, Berlin (1995). Nevertheless, the present invention contemplates the transformation of plants with a hyperthermophilic processing enzyme in accord with known transforming methods. *Also see*, Weissinger et al., Annual Rev. Genet., 22:421 (1988); Sanford et al., Particulate Science and

Technology, 5:27 (1987) (onion); Christou et al., Plant Physiol., 87:671 (1988) (soybean); McCabe et al., Bio/Technology, 6:923 (1988) (soybean); Datta et al., Bio/Technology, 8:736 (1990) (rice); Klein et al., Proc. Natl. Acad. Sci. USA, 85:4305 (1988) (maize); Klein et al., Bio/Technology, 6:559 (1988) (maize); Klein et al., Plant Physiol., 91:440 (1988) (maize); Fromm et al., Bio/Technology, 8:833 (1990) (maize); and Gordon-Kamm et al., Plant Cell, 2, 603 (1990) (maize); Svab et al., Proc. Natl. Acad. Sci. USA, 87:8526 (1990) (tobacco chloroplast); Koziel et al., Biotechnology, 11:194 (1993) (maize); Shimamoto et al., Nature, 338:274 (1989) (rice); Christou et al., Biotechnology, 9:957 (1991) (rice); European Patent Application EP 0 332 581 (orchardgrass and other Pooideae); Vasil et al., Biotechnology, 11:1553 (1993) (wheat); Weeks et al., Plant Physiol., 102:1077 (1993) (wheat). Methods in Molecular Biology, 82. Arabidopsis Protocols Ed. Martinez-Zapater and Salinas 1998 Humana Press (Arabidopsis).

Transformation of plants can be undertaken with a single DNA molecule or multiple DNA molecules (i.e., co-transformation), and both these techniques are suitable for use with the expression cassettes and constructs of the present invention. Numerous transformation vectors are available for plant transformation, and the expression cassettes of this invention can be used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation.

Ultimately, the most desirable DNA segments for introduction into a monocot genome may be homologous genes or gene families which encode a desired trait (e.g., hydrolysis of proteins, lipids or polysaccharides) and which are introduced under the control of novel promoters or enhancers, etc., or perhaps even homologous or tissue specific (e.g., root-, collar/sheath-, whorl-, stalk-, earshank-, kernel- or leaf-specific) promoters or control elements. Indeed, it is envisioned that a particular use of the present invention will be the targeting of a gene in a constitutive manner or in an inducible manner.

#### Examples of Suitable Transformation Vectors

Numerous transformation vectors available for plant transformation are known to those of ordinary skill in the plant transformation arts, and the genes pertinent to this invention can be used in conjunction with any such vectors known in the art. The selection of vector will depend upon the preferred transformation technique and the target species for transformation.



a. Vectors Suitable for *Agrobacterium* Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). Below, the construction of two typical vectors suitable for *Agrobacterium* transformation is described.

pCIB200 and pCIB2001

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and are constructed in the following manner. pTJS75kan is created by NarI digestion of pTJS75 (Schmidhauser & Helinski, J. Bacteriol., 164: 446 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an AccI fragment from pUC4K carrying an NPTII (Messing & Vierra, Gene, 19: 259 (1982); Bevan et al., Nature, 304: 184 (1983); McBride et al., Plant Molecular Biology, 14: 266 (1990)). XhoI linkers are ligated to the EcoRV fragment of PCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein et al., Gene, 53: 153 (1987)), and the XhoI-digested fragment are cloned into Sall-digested pTJS75kan to create pCIB200 (see also EP 0 332 104, example 19). pCIB200 contains the following unique polylinker restriction sites: EcoRI, SstI, KpnI, BglII, XbaI, and Sall. pCIB2001 is a derivative of pCIB200 created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are EcoRI, SstI, KpnI, BglII, XbaI, Sall, MluI, BclI, AvrII, ApaI, HpaI, and StuI. pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

pCIB10 and Hygromycin Selection Derivatives thereof:

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants and T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium*. Its construction is described by Rothstein et al. (Gene, 53: 153 (1987)). Various derivatives of pCIB10 are constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz et al. (Gene, 25: 179 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717).

b. Vectors Suitable for non-*Agrobacterium* Transformation

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques that do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (e.g., PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. Non-limiting examples of the construction of typical vectors suitable for non-*Agrobacterium* transformation is further described.

pCIB3064

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide basta (or phosphinothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in the PCT published application WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites are mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites SspI and PvuII. The new restriction sites are 96 and 37 bp away from the unique SalI site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 is designated pCIB3025. The GUS gene is then excised from pCIB3025 by digestion with SalI and SacI, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 may be obtained from the John Innes Centre, Norwich and the a 400 bp *SmaI* fragment containing the *bar* gene from *Streptomyces viridochromogenes* is excised and inserted into the

*HpaI* site of pCIB3060 (Thompson et al., EMBO J, 6: 2519 (1987)). This generated pCIB3064, which comprises the *bar* gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene for ampicillin resistance (for selection in *E. coli*) and a polylinker with the unique sites *SphI*, *PstI*, *HindIII*, and *BamHI*. This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

#### pSOG19 and pSOG35:

The plasmid pSOG35 is a transformation vector that utilizes the *E. coli* gene dihydrofolate reductase (DHFR) as a selectable marker conferring resistance to methotrexate. PCR is used to amplify the 35S promoter (-800 bp), intron 6 from the maize *Adh1* gene (-550 bp) and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250-bp fragment encoding the *E. coli* dihydrofolate reductase type II gene is also amplified by PCR and these two PCR fragments are assembled with a *SacI*-*PstI* fragment from pB1221 (Clontech) which comprises the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generates pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generates the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have *HindIII*, *SphI*, *PstI* and *EcoRI* sites available for the cloning of foreign substances.

#### c. Vector Suitable for Chloroplast Transformation

For expression of a nucleotide sequence of the present invention in plant plastids, plastid transformation vector pPH143 (WO 97/32011, example 36) is used. The nucleotide sequence is inserted into pPH143 thereby replacing the PROTOX coding sequence. This vector is then used for plastid transformation and selection of transformants for spectinomycin resistance. Alternatively, the nucleotide sequence is inserted in pPH143 so that it replaces the *aadH* gene. In this case, transformants are selected for resistance to PROTOX inhibitors.

### **Plant Hosts Subject to Transformation Methods**

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a construct of the present invention. The term

organogenesis means a process by which shoots and roots are developed sequentially from meristematic centers while the term embryogenesis means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include differentiated and undifferentiated tissues or plants, including but not limited to leaf disks, roots, stems, shoots, leaves, pollen, seeds, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem), tumor tissue, and various forms of cells and culture such as single cells, protoplast, embryos, and callus tissue. The plant tissue may be in plants or in organ, tissue or cell culture.

Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the expression cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as npt II) can be associated with the expression cassette to assist in breeding.

The present invention may be used for transformation of any plant species, including monocots or dicots, including, but not limited to, corn (*Zea mays*), Brassica sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those Brassica species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea*

batatus), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, woody plants such as conifers and deciduous trees, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, soybean, sorghum, sugarcane, rapeseed, clover, carrot, and *Arabidopsis thaliana*.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*), Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc. Legumes include, but are not limited to, *Arachis*, e.g., peanuts, *Vicia*, e.g., crown vetch, hairy vetch, adzuki bean, mung bean, and chickpea, *Lupinus*, e.g., lupine, trifolium, *Phaseolus*, e.g., common bean and lima bean, *Pisum*, e.g., field bean, *Melilotus*, e.g., clover, *Medicago*, e.g., alfalfa, *Lotus*, e.g., trefoil, lens, e.g., lentil, and false indigo. Preferred forage and turf grass for use in the methods of the



invention include alfalfa, orchard grass, tall fescue, perennial ryegrass, creeping bent grass, and redtop.

Preferably, plants of the present invention include crop plants, for example, corn, alfalfa, sunflower, Brassica, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, barley, rice, tomato, potato, squash, melons, legume crops, etc. Other preferred plants include Liliopsida and Panicoideae.

Once a desired DNA sequence has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques.

Below are descriptions of representative techniques for transforming both dicotyledonous and monocotyledonous plants, as well as a representative plastid transformation technique.

a. Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques that do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski et al., EMBO J, 3: 2717 (1984), Potrykus et al., Mol. Gen. Genet., 199: 169 (1985), Reich et al., Biotechnology, 4: 1001 (1986), and Klein et al., Nature, 327: 70 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

*Agrobacterium*-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (e.g. pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend on the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (e.g., strain CIB542 for pCIB200 and pCIB2001 (Uknes et al., Plant Cell, 5: 159 (1993))). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a

plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res., 16: 9877 (1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

The vectors may be introduced to plant cells in known ways. Preferred cells for transformation include *Agrobacterium*, monocot cells and dicots cells, including Liliopsida cells and Panicoideae cells. Preferred monocot cells are cereal cells, e.g., maize (corn), barley, and wheat, and starch accumulating dicot cells, e.g., potato.

Another approach to transforming a plant cell with a gene involves propelling inert or biologically active particles at plant tissues and cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and afford incorporation within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the desired gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into plant cell tissue.

b. Transformation of Monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using polyethylene glycol (PEG) or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (i.e., co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complete vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the

selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher et al., Biotechnology, 4: 1093 1986)).

Patent Applications EP 0 292 435, EP 0 392 225, and WO 93/07278 describe techniques for the preparation of callus and protoplasts from an elite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm et al. (Plant Cell, 2: 603 (1990)) and Fromm et al. (Biotechnology, 8: 833 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, WO 93/07278 and Koziel et al. (Biotechnology, 11: 194 (1993)) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang et al., Plant Cell Rep, 7: 379 (1988); Shimamoto et al., Nature, 338: 274 (1989); Datta et al., Biotechnology, 8: 736 (1990)). Both types are also routinely transformable using particle bombardment (Christou et al., Biotechnology, 9: 957 (1991)). Furthermore, WO 93/21335 describes techniques for the transformation of rice via electroporation. Patent Application EP 0 332 581 describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation has been described by Vasil et al. (Biotechnology, 10: 667 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil et al. (Biotechnology, 11: 1553 (1993)) and Weeks et al. (Plant Physiol., 102: 1077 (1993)) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with

3% sucrose (Murashiga & Skoog, Physiologia Plantarum, 15: 473 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos, which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (i.e., induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 hours and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of about 1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 hours (still on osmoticum). After 24 hours, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contain half-strength MS, 2% sucrose, and the same concentration of selection agent.

Transformation of monocotyledons using *Agrobacterium* has also been described. See, WO 94/00977 and U.S. Patent No. 5,591,616, both of which are incorporated herein by reference.

c. Transformation of Plastids

Seeds of *Nicotiana tabacum* c.v. 'Xanthi nc' are germinated seven per plate in a 1" circular array on T agar medium and bombarded 12-14 days after sowing with 1 µm tungsten particles (M10, Biorad, Hercules, CA) coated with DNA from plasmids pPH143 and pPH145 essentially as described (Svab and Maliga, PNAS, 90:913 (1993)). Bombarded seedlings are incubated on T medium for two days after which leaves are excised and placed abaxial side up in bright light (350-500 µmol photons/m<sup>2</sup>/s) on plates of RMOP medium (Svab, Hajdukiewicz and Maliga, PNAS, 87:8526 (1990)) containing 500 µg/ml spectinomycin dihydrochloride (Sigma, St. Louis, MO). Resistant shoots appearing underneath the bleached leaves three to eight weeks

after bombardment are subcloned onto the same selective medium, allowed to form callus, and secondary shoots isolated and subcloned. Complete segregation of transformed plastid genome copies (homoplasmy) in independent subclones is assessed by standard techniques of Southern blotting (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (1989)). BamHI/EcoRI-digested total cellular DNA (Mettler, I. J. Plant Mol Biol Reporter, 5:346 (1987)) is separated on 1% Tris-borate (TBE) agarose gels, transferred to nylon membranes (Amersham) and probed with <sup>32</sup>P-labeled random primed DNA sequences corresponding to a 0.7 kb BamHI/HindIII DNA fragment from pC8 containing a portion of the *rps7/12* plastid targeting sequence. Homoplasmic shoots are rooted aseptically on spectinomycin-containing MS/IBA medium (McBride et al., PNAS, 91:7301 (1994)) and transferred to the greenhouse.

### **Production and Characterization of Stably Transformed Plants**

Transformed plant cells are then placed in an appropriate selective medium for selection of transgenic cells, which are then grown to callus. Shoots are grown from callus and plantlets generated from the shoot by growing in rooting medium. The various constructs normally will be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a biocide (particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, herbicide, or the like). The particular marker used will allow for selection of transformed cells as compared to cells lacking the DNA which has been introduced. Components of DNA constructs, including transcription/expression cassettes of this invention, may be prepared from sequences, which are native (endogenous) or foreign (exogenous) to the host. By "foreign" it is meant that the sequence is not found in the wild-type host into which the construct is introduced. Heterologous constructs will contain at least one region, which is not native to the gene from which the transcription-initiation-region is derived.

To confirm the presence of the transgenes in transgenic cells and plants, a Southern blot analysis can be performed using methods known to those skilled in the art. Integration of a polynucleic acid segment into the genome can be detected and quantitated by Southern blot, since they can be readily distinguished from constructs containing the segments through use of appropriate restriction enzymes. Expression products of the transgenes can be detected in any of



a variety of ways, depending upon the nature of the product, and include Western blot and enzyme assay. One particularly useful way to quantitate protein expression and to detect replication in different plant tissues is to use a reporter gene, such as GUS. Once transgenic plants have been obtained, they may be grown to produce plant tissues or parts having the desired phenotype. The plant tissue or plant parts may be harvested, and/or the seed collected. The seed may serve as a source for growing additional plants with tissues or parts having the desired characteristics.

The invention thus provides a transformed plant or plant part, such as an ear, seed, fruit, grain, stover, chaff, or bagasse comprising at least one polynucleotide, expression cassette or vector of the invention, methods of making such a plant and methods of using such a plant or a part thereof. The transformed plant or plant part expresses a processing enzyme, optionally localized in a particular cellular or subcellular compartment of a certain tissue or in developing grain. For instance, the invention provides a transformed plant part comprising at least one starch processing enzyme present in the cells of the plant, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one starch processing enzyme. The processing enzyme does not act on the target substrate unless activated by methods such as heating, grinding, or other methods, which allow the enzyme to contact the substrate under conditions where the enzyme is active

### **Exemplary Methods of the Present Invention**

The self-processing plants and plant parts of the present invention may be used in various methods employing the processing enzymes (mesophilic, thermophilic, or hyperthermophilic) expressed and activated therein. In accordance with the present invention, a transgenic plant part obtained from a transgenic plant the genome of which is augmented with at least one processing enzyme, is placed under conditions in which the processing enzyme is expressed and activated. Upon activation, the processing enzyme is activated and functions to act on the substrate in which it normally acts to obtain the desired result. For example, the starch-processing enzymes act upon starch to degrade, hydrolyze, isomerize, or otherwise modify to obtain the desired result upon activation. Non-starch processing enzymes may be used to disrupt the plant cell membrane in order to facilitate the extraction of starch, lipids, amino acids, or other products

from the plants. Moreover, non-hyperthermophilic and hyperthermophilic enzymes may be used in combination in the self-processing plant or plant parts of the present invention. For example, a mesophilic non-starch degrading enzyme may be activated to disrupt the plant cell membrane for starch extraction, and subsequently, a hyperthermophilic starch-degrading enzyme may then be activated in the self-processing plant to degrade the starch.

Enzymes expressed in grain can be activated by placing the plant or plant part containing them in conditions in which their activity is promoted. For example, one or more of the following techniques may be used: The plant part may be contacted with water, which provides a substrate for a hydrolytic enzyme and thus will activate the enzyme. The plant part may be contacted with water which will allow enzyme to migrate from the compartment into which it was deposited during development of the plant part and thus to associate with its substrate. Movement of the enzyme is possible because compartmentalization is breached during maturation, drying of grain and re-hydration. The intact or cracked grain may be contacted with water which will allow enzyme to migrate from the compartment into which it was deposited during development of the plant part and thus to associate with its substrate. Enzymes can also be activated by addition of an activating compound. For example, a calcium-dependent enzyme can be activated by addition of calcium. Other activating compounds may be determined by those skilled in the art. Enzymes can be activated by removal of an inactivator. For example, there are known peptide inhibitors of amylase enzymes, the amylase could be co-expressed with an amylase inhibitor and then activated by addition of a protease. Enzymes can be activated by alteration of pH to one at which the enzyme is most active. Enzymes can also be activated by increasing temperature. An enzyme generally increases in activity up to the maximal temperature for that enzyme. A mesophilic enzyme will increase in activity from the level of activity at ambient temperature up to the temperature at which it loses activity which is typically less than or equal to 70 °C. Similarly thermophilic and hyperthermophilic enzymes can also be activated by increasing temperature. Thermophilic enzymes can be activated by heating to temperatures up to the maximal temperature of activity or of stability. For a thermophilic enzyme the maximal temperatures of stability and activity will generally be between 70 and 85 °C. Hyperthermophilic enzymes will have the even greater relative activation than mesophilic or

thermophilic enzymes because of the greater potential change in temperature from 25 °C up to 85 °C to 95 °C or even 100 °C. The increased temperature may be achieved by any method, for example by heating such as by baking, boiling, heating, steaming, electrical discharge or any combination thereof. Moreover, in plants expressing mesophilic or thermophilic enzyme(s), activation of the enzyme may be accomplished by grinding, thereby allowing the enzyme to contact the substrate.

The optimal conditions, e.g., temperature, hydration, pH, etc, may be determined by one having skill in the art and may depend upon the individual enzyme being employed and the desired application of the enzyme.

The present invention further provides for the use of exogenous enzymes that may assist in a particular process. For example, the use of a self-processing plant or plant part of the present invention may be used in combination with an exogenously provided enzyme to facilitate the reaction. As an example, transgenic  $\alpha$ -amylase corn may be used in combination with other starch-processing enzymes, such as pullulanase,  $\alpha$ -glucosidase, glucose isomerase, mannanases, hemicellulases, etc., to hydrolyze starch or produce ethanol. In fact, it has been found that combinations of the transgenic  $\alpha$ -amylase corn with such enzymes has unexpectedly provided superior degrees of starch conversion relative to the use of transgenic  $\alpha$ -amylase corn alone.

Example of suitable methods contemplated herein are provided.

a. Starch Extraction From Plants

The invention provides for a method of facilitating the extraction of starch from plants. In particular, at least one polynucleotide encoding a processing enzyme that disrupts the physically restraining matrix of the endosperm (cell walls, non-starch polysaccharide, and protein matrix) is introduced to a plant so that the enzyme is preferably in close physical proximity to starch granules in the plant. In this embodiment of the invention, transformed plants express one or more protease, glucanase, xylanase, thioredoxin/thioredoxin reductase, cellulase, phytase, lipase, beta glucosidase, esterase and the like, but not enzymes that have any starch degrading activity, so as to maintain the integrity of the starch granules. The expression of these enzymes in a plant part such as grain thus improves the process characteristics of grain. The processing enzyme may be mesophilic, thermophilic, or hyperthermophilic. In one example,

grain from a transformed plant of the invention is heat dried, likely inactivating non-hyperthermophilic processing enzymes and improving seed integrity. Grain (or cracked grain) is steeped at low temperatures or high temperatures (where time is of the essence) with high or low moisture content or conditions (see Primary Cereal Processing, Gordon and Willm, eds., pp. 319-337 (1994), the disclosure of which is incorporated herein), with or without sulphur dioxide. Upon reaching elevated temperatures, optionally at certain moisture conditions, the integrity of the endosperm matrix is disrupted by activating the enzymes, e.g., proteases, xylanases, phytase or glucanases which degrade the proteins and non-starch polysaccharides present in the endosperm leaving the starch granule therein intact and more readily recoverable from the resulting material. Further, the proteins and non-starch polysaccharides in the effluent are at least partially degraded and highly concentrated, and so may be used for improved animal feed, food, or as media components for the fermentation of microorganisms. The effluent is considered a corn-steep liquor with improved composition.

Thus, the invention provides a method to prepare starch granules. The method comprises treating grain, for example cracked grain, which comprises at least one non-starch processing enzyme under conditions which activate the at least one enzyme, yielding a mixture comprising starch granules and non-starch degradation products, e.g., digested endosperm matrix products. The non-starch processing enzyme may be mesophilic, thermophilic, or hyperthermophilic. After activation of the enzyme, the starch granules are separated from the mixture. The grain is obtained from a transformed plant, the genome of which comprises (is augmented with) an expression cassette encoding the at least one processing enzyme. For example, the processing enzyme may be a protease, glucanase, xylanase, phytase, thioredoxin/thioredoxin reductase, esterase cellulase, lipase, or a beta glucosidase. The processing enzyme may be hyperthermophilic. The grain can be treated under low or high moisture conditions, in the presence or absence of sulfur dioxide. Depending on the activity and expression level of the processing enzyme in the grain from the transgenic plant, the transgenic grain may be mixed with commodity grain prior to or during processing. Also provided are products obtained by the method such as starch, non-starch products and improved steepwater comprising at least one additional component.

b. Starch-Processing Methods

Transformed plants or plant parts of the present invention may comprise starch-degrading enzymes as disclosed herein that degrade starch granules to dextrans, other modified starches, or hexoses (*e.g.*,  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucoamylase, amylopullulanase) or convert glucose into fructose (*e.g.*, glucose isomerase). Preferably, the starch-degrading enzyme is selected from  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase, neopullulanase, amylopullulanase, glucose isomerase, and combinations thereof is used to transform the grain. Moreover, preferably, the enzyme is operably linked to a promoter and to a signal sequence that targets the enzyme to the starch granule, an amyloplast, the apoplast, or the endoplasmic reticulum. Most preferably, the enzyme is expressed in the endosperm, and particularly, corn endosperm, and localized to one or more cellular compartments, or within the starch granule itself. The preferred plant part is grain. Preferred plant parts are those from corn, wheat, barley, rye, oat, sugar cane, or rice.

In accordance with one starch-degrading method of the present invention, the transformed grain accumulates the starch-degrading enzyme in starch granules, is steeped at conventional temperatures of 50°C-60°C, and wet-milled as is known in the art. Preferably, the starch-degrading enzyme is hyperthermophilic. Because of sub-cellular targeting of the enzyme to the starch granule, or by virtue of the association of the enzyme with the starch granule, by contacting the enzyme and starch granule during the wet-milling process at the conventional temperatures, the processing enzyme is co-purified with the starch granules to obtain the starch granules/enzyme mixture. Subsequent to the recovery of the starch granules/enzyme mixture, the enzyme is then activated by providing favorable conditions for the activity of the enzyme. For example, the processing may be performed in various conditions of moisture and/or temperature to facilitate the partial (in order to make derivatized starches or dextrans) or complete hydrolysis of the starch into hexoses. Syrups containing high dextrose or fructose equivalents are obtained in this manner. This method effectively reduces the time, energy, and enzyme costs and the efficiency with which starch is converted to the corresponding hexose, and the efficiency of the production of products, like high sugar steepwater and higher dextrose equivalent syrups, are increased.



In another embodiment, a plant, or a product of the plant such as a fruit or grain, or flour made from the grain that expresses the enzyme is treated to activate the enzyme and convert polysaccharides expressed and contained within the plant into sugars. Preferably, the enzyme is fused to a signal sequence that targets the enzyme to a starch granule, an amyloplast, the apoplast or to the endoplasmic reticulum as disclosed herein. The sugar produced may then be isolated or recovered from the plant or the product of the plant. In another embodiment, a processing enzyme able to convert polysaccharides into sugars is placed under the control of an inducible promoter according to methods known in the art and disclosed herein. The processing enzyme may be mesophilic, thermophilic or hyperthermophilic. The plant is grown to a desired stage and the promoter is induced causing expression of the enzyme and conversion of the polysaccharides, within the plant or product of the plant, to sugars. Preferably the enzyme is operably linked to a signal sequence that targets the enzyme to a starch granule, an amyloplast, an apoplast or to the endoplasmic reticulum. In another embodiment, a transformed plant is produced that expresses a processing enzyme able to convert starch into sugar. The enzyme is fused to a signal sequence that targets the enzyme to a starch granule within the plant. Starch is then isolated from the transformed plant that contains the enzyme expressed by the transformed plant. The enzyme contained in the isolated starch may then be activated to convert the starch into sugar. The enzyme may be mesophilic, thermophilic, or hyperthermophilic. Examples of hyperthermophilic enzymes able to convert starch to sugar are provided herein. The methods may be used with any plant which produces a polysaccharide and that can express an enzyme able to convert a polysaccharide into sugars or hydrolyzed starch product such as dextrin, maltooligosaccharide, glucose and/or mixtures thereof.

The invention provides a method to produce dextrans and altered starches from a plant, or a product from a plant, that has been transformed with a processing enzyme which hydrolyses certain covalent bonds of a polysaccharide to form a polysaccharide derivative. In one embodiment, a plant, or a product of the plant such as a fruit or grain, or flour made from the grain that expresses the enzyme is placed under conditions sufficient to activate the enzyme and convert polysaccharides contained within the plant into polysaccharides of reduced molecular weight. Preferably, the enzyme is fused to a signal sequence that targets the enzyme to a starch granule, an amyloplast, the apoplast or to the endoplasmic reticulum as disclosed herein. The

dextrin or derivative starch produced may then be isolated or recovered from the plant or the product of the plant. In another embodiment, a processing enzyme able to convert polysaccharides into dextrans or altered starches is placed under the control of an inducible promoter according to methods known in the art and disclosed herein. The plant is grown to a desired stage and the promoter is induced causing expression of the enzyme and conversion of the polysaccharides, within the plant or product of the plant, to dextrans or altered starches. Preferably the enzyme is  $\alpha$ -amylase, pullulanase, iso or neo-pullulanase and is operably linked to a signal sequence that targets the enzyme to a starch granule, an amyloplast, the apoplast or to the endoplasmic reticulum. In one embodiment, the enzyme is targeted to the apoplast or to the endoreticulum. In yet another embodiment, a transformed plant is produced that expresses an enzyme able to convert starch into dextrans or altered starches. The enzyme is fused to a signal sequence that targets the enzyme to a starch granule within the plant. Starch is then isolated from the transformed plant that contains the enzyme expressed by the transformed plant. The enzyme contained in the isolated starch may then be activated under conditions sufficient for activation to convert the starch into dextrans or altered starches. Examples of hyperthermophilic enzymes, for example, able to convert starch to hydrolyzed starch products are provided herein. The methods may be used with any plant which produces a polysaccharide and that can express an enzyme able to convert a polysaccharide into sugar.

In another embodiment, grain from transformed plants of the invention that accumulate starch-degrading enzymes that degrade linkages in starch granules to dextrans, modified starches or hexose (*e.g.*,  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, glucoamylase, amylopullulanase) is steeped under conditions favoring the activity of the starch degrading enzyme for various periods of time. The resulting mixture may contain high levels of the starch-derived product. The use of such grain: 1) eliminates the need to mill the grain, or otherwise process the grain to first obtain starch granules, 2) makes the starch more accessible to enzymes by virtue of placing the enzymes directly within the endosperm tissue of the grain, and 3) eliminates the need for microbially produced starch-hydrolyzing enzymes. Thus, the entire process of wet-milling prior to hexose recovery is eliminated by simply heating grain, preferably corn grain, in the presence of water to allow the enzymes to act on the starch.

This process can also be employed for the production of ethanol, high fructose syrups, hexose (glucose) containing fermentation media, or any other use of starch that does not require the refinement of grain components.

The invention further provides a method of preparing dextrin, maltooligosaccharides, and/or sugar involving treating a plant part comprising starch granules and at least one starch processing enzyme under conditions so as to activate the at least one enzyme thereby digesting starch granules to form an aqueous solution comprising sugars. The plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one processing enzyme. The aqueous solution comprising dextrans, maltooligosaccharides, and/or sugar is then collected. In one embodiment, the processing enzyme is  $\alpha$ -amylase,  $\alpha$ -glucosidase, pullulanase, glucoamylase, amylopullulanase, glucose isomerase, or any combination thereof. Preferably, the enzyme is hyperthermophilic. In another embodiment, the method further comprises isolating the dextrans, maltooligosaccharides, and/or sugar.

c. Improved Corn Varieties

The invention also provides for the production of improved corn varieties (and varieties of other crops) that have normal levels of starch accumulation, and accumulate sufficient levels of amylolytic enzyme(s) in their endosperm, or starch accumulating organ, such that upon activation of the enzyme contained therein, such as by boiling or heating the plant or a part thereof in the case of a hyperthermophilic enzyme, the enzyme(s) is activated and facilitates the rapid conversion of the starch into simple sugars. These simple sugars (primarily glucose) will provide sweetness to the treated corn. The resulting corn plant is an improved variety for dual use as a grain

producing hybrid and as sweet corn. Thus, the invention provides a method to produce hyper-sweet corn, comprising treating transformed corn or a part thereof, the genome of which is augmented with and expresses in endosperm an expression cassette comprising a promoter operably linked to a first polynucleotide encoding at least one amylolytic enzyme, conditions which activate the at least one enzyme so as to convert polysaccharides in the corn into sugar, yielding hypersweet corn. The promoter may be a constitutive promoter, a seed-

specific promoter, or an endosperm-specific promoter which is linked to a polynucleotide sequence which encodes a processing enzyme such as  $\alpha$ -amylase, e.g., one comprising SEQ ID NO: 13, 14, or 16. Preferably, the enzyme is hyperthermophilic. In one embodiment, the expression cassette further comprises a second polynucleotide which encodes a signal sequence operably linked to the enzyme encoded by the first polynucleotide. Exemplary signal sequences in this embodiment of the invention direct the enzyme to apoplast, the endoplasmic reticulum, a starch granule, or to an amyloplast. The corn plant is grown such that the ears with kernels are formed and then the promoter is induced to cause the enzyme to be expressed and convert polysaccharide contained within the plant into sugar.

d. Self-Fermenting Plants

In another embodiment of the invention, plants, such as corn, rice, wheat, or sugar cane are engineered to accumulate large quantities of processing enzymes in their cell walls, e.g., xylanases, cellulases, hemicellulases, glucanases, pectinases, lipases, esterases, beta glucosidases, phytases, proteases and the like (non-starch polysaccharide degrading enzymes). Following the harvesting of the grain component (or sugar in the case of sugar cane), the stover, chaff, or bagasse is used as a source of the enzyme, which was targeted for expression and accumulation in the cell walls, and as a source of biomass. The stover (or other left-over tissue) is used as a feedstock in a process to recover fermentable sugars. The process of obtaining the fermentable sugars consists of activating the non-starch polysaccharide degrading enzyme. For example, activation may comprise heating the plant tissue in the presence of water for periods of time adequate for the hydrolysis of the non-starch polysaccharide into the resulting sugars. Thus, this self-processing stover produces the enzymes required for conversion of polysaccharides into monosaccharides, essentially at no incremental cost as they are a component of the feedstock. Further, the temperature-dependent enzymes have no detrimental effects on plant growth and development, and cell wall targeting, even targeting into polysaccharide microfibrils by virtue of cellulose/xylose binding domains fused to the protein, improves the accessibility of the substrate to the enzyme.

Thus, the invention also provides a method of using a transformed plant part comprising at least one non-starch polysaccharide processing enzyme in the cell wall of the cells of the plant

part. The method comprises treating a transformed plant part comprising at least one non-starch polysaccharide processing enzyme under conditions which activate the at least one enzyme thereby digesting starch granules to form an aqueous solution comprising sugars, wherein the plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one non-starch polysaccharide processing enzyme; and collecting the aqueous solution comprising the sugars. The invention also includes a transformed plant or plant part comprising at least one non-starch polysaccharide processing enzyme present in the cell or cell wall of the cells of the plant or plant part. The plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one non-starch processing enzyme, e.g., a xylanase, cellulase, glucanase, pectinase, lipase, esterase, beta glucosidase, phytase, protease or any combination thereof.



e. Aqueous Phase High In Protein and Sugar Content

In yet another embodiment, proteases and lipases are engineered to accumulate in seeds, e.g., soybean seeds. After activation of the protease or lipase, such as, for example, by heating, these enzymes in the seeds hydrolyze the lipid and storage proteins present in soybeans during processing. Soluble products comprising amino acids, which can be used as feed, food or fermentation media, and fatty acids, can thus be obtained. Polysaccharides are typically found in the insoluble fraction of processed grain. However, by combining polysaccharide degrading enzyme expression and accumulation in seeds, proteins and polysaccharides can be hydrolyzed and are found in the aqueous phase. For example, zeins from corn and storage protein and non-starch polysaccharides from soybean can be solubilized in this manner. Components of the aqueous and hydrophobic phases can be easily separated by extraction with organic solvent or supercritical carbon dioxide. Thus, what is provided is a method for producing an aqueous extract of grain that contains higher levels of protein, amino acids, sugars or saccharides.

f. Self-Processing Fermentation

The invention provides a method to produce ethanol, a fermented beverage, or other fermentation-derived product(s). The method involves obtaining a plant, or the product or part of a plant, or plant derivative such as grain flour, wherein a processing enzyme that converts polysaccharides into sugar is expressed. The plant, or product thereof, is treated such that sugar is produced by conversion of the polysaccharide as described above. The sugars and other components of the plant are then fermented to form ethanol or a fermented beverage, or other fermentation-derived products, according to methods known in the art. See, for example, U.S. Patent No.: 4,929,452. Briefly the sugar produced by conversion of polysaccharides is incubated with yeast under conditions that promote conversion of the sugar into ethanol. A suitable yeast includes high alcohol-tolerant and high-sugar tolerant strains of yeast, such as, for example, the yeast, *S. cerevisiae* ATCC No. 20867. This strain was deposited with the American Type Culture Collection, Rockville, MD, on Sept. 17, 1987 and assigned ATCC No. 20867. The fermented product or fermented beverage may then be distilled to isolate ethanol or a distilled beverage, or the fermentation product otherwise recovered. The plant used in this method may be any plant that contains a polysaccharide and is able to express an enzyme of the invention. Many such plants are disclosed herein. Preferably the plant is one that is grown commercially. More

preferably the plant is one that is normally used to produce ethanol or fermented beverages, or fermented products, such as, for example, wheat, barley, corn, rye, potato, grapes or rice.

The method comprises treating a plant part comprising at least one polysaccharide processing enzyme under conditions to activate the at least one enzyme thereby digesting polysaccharide in the plant part to form fermentable sugar. The polysaccharide processing enzyme may be mesophilic, thermophilic, or hyperthermophilic. The plant part is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one polysaccharide processing enzyme. Plant parts for this embodiment of the invention include, but are not limited to, grain, fruit, seed, stalk, wood, vegetable or root. Plants include but are not limited to oat, barley, wheat, berry, grape, rye, corn, rice, potato, sugar beet, sugar cane, pineapple, grass and tree. The plant part may be combined with commodity grain or other commercially available substrates; the source of the substrate for processing may be a source other than the self-processing plant. The fermentable sugar is then incubated under conditions that promote the conversion of the fermentable sugar into ethanol, e.g., with yeast and/or other microbes. In an embodiment, the plant part is derived from corn transformed with  $\alpha$ -amylase, which has been found to reduce the amount of time and cost of fermentation.

It has been found that the amount of residual starch is reduced when transgenic corn made in accordance with the present invention expressing a thermostable  $\alpha$ -amylase, for example, is used in fermentation. This indicates that more starch is solubilized during fermentation. The reduced amount of residual starch results in the distillers' grains having higher protein content by weight and higher value. Moreover, the fermentation of the transgenic corn of the present invention allows the liquefaction process to be performed at a lower pH, resulting in savings in the cost of chemicals used to adjust the pH, at a higher temperature, e.g., greater than 85°C, preferably, greater than 90°C, more preferably, 95°C or higher, resulting in shorter liquefaction times and more complete solubilization of starch, and reduction of liquefaction times, all resulting in efficient fermentation reactions with higher yields of ethanol.

Moreover, it has been found that contacting conventional plant parts with even a small portion of the transgenic plant made in accordance with the present invention may reduce the fermentation time and costs associated therewith. As such, the present invention relates to the reduction in the fermentation time for plants comprising subjecting a transgenic plant part from a

plant comprising a polysaccharide processing enzyme that converts polysaccharides into sugar relative to the use of a plant part not comprising the polysaccharide processing enzyme.

g. Raw Starch Processing Enzymes And Polynucleotides Encoding Them

A polynucleotide encoding a mesophilic processing enzyme(s) is introduced into a plant or plant part. In an embodiment, the polynucleotide of the present invention is a maize-optimized polynucleotide such as provided in SEQ ID NOs: 48, 50, and 59, encoding a glucoamylase, such as provided in SEQ ID NOs: 47, and 49. In another embodiment, the polynucleotide of the present invention is a maize-optimized polynucleotide such as provided in SEQ ID NO: 52, encoding an alpha-amylase, such as provided in SEQ ID NO: 51. Moreover, fusion products of processing enzymes are further contemplated. In one embodiment, the polynucleotide of the present invention is a maize-optimized polynucleotide such as provided in SEQ ID NO: 46, encoding an alpha-amylase and glucoamylase fusion, such as provided in SEQ ID NO: 45. Combinations of processing enzymes are further envisioned by the present invention. For example, a combination of starch-processing enzymes and non-starch processing enzymes is contemplated herein. Such combinations of processing enzymes may be obtained by employing the use of multiple gene constructs encoding each of the enzymes. Alternatively, the individual transgenic plants stably transformed with the enzymes may be crossed by known methods to obtain a plant containing both enzymes. Another method includes the use of exogenous enzyme(s) with the transgenic plant.

The source of the starch-processing and non-starch processing enzymes may be isolated or derived from any source and the polynucleotides corresponding thereto may be ascertained by one having skill in the art. The  $\alpha$ -amylase may be derived from *Aspergillus* (e.g., *Aspergillus shirousami* and *Aspergillus niger*), *Rhizopus* (eg., *Rhizopus oryzae*), and plants such as corn, barley, and rice. The glucoamylase may be derived from *Aspergillus* (e.g., *Aspergillus shirousami* and *Aspergillus niger*), *Rhizopus* (eg., *Rhizopus oryzae*), and *Thermoanaerobacter* (eg., *Thermoanaerobacter thermosaccharolyticum*).

In another embodiment of the invention, the polynucleotide encodes a mesophilic starch-processing enzyme that is operably linked to a maize-optimized polynucleotide such as provided in SEQ ID NO: 54, encoding a raw starch binding domain, such as provided in SEQ ID NO: 53.

In another embodiment, a tissue-specific promoter includes the endosperm-specific promoters such as the maize  $\gamma$ -zein promoter (exemplified by SEQ ID NO:12) or the maize ADP-gpp promoter (exemplified by SEQ ID NO:11, which includes a 5' untranslated and an intron sequence) or a Q protein promoter (exemplified by SEQ ID NO: 98) or a rice glutelin promoter (exemplified by SEQ ID NO: 67) . Thus, the present invention includes an isolated polynucleotide comprising a promoter comprising SEQ ID NO: 11, 12, 67, or 98, a polynucleotide which hybridizes to the complement thereof under low stringency hybridization conditions, or a fragment thereof which has promoter activity, e.g., at least 10%, and preferably at least 50%, the activity of a promoter having SEQ ID NO:11, 12, 67 or 98.

In one embodiment, the product from a starch-hydrolysis gene, such as  $\alpha$ -amylase, glucoamylase, or  $\alpha$ -amylase/glucoamylase fusion may be targeted to a particular organelle or location such as the endoplasmic reticulum or apoplast, rather than to the cytoplasm. This is exemplified by the use of the maize  $\gamma$ -zein N-terminal signal sequence (SEQ ID NO:17), which confers apoplast-specific targeting of proteins, and the use of the  $\gamma$ -zein N-terminal signal sequence (SEQ ID NO:17) which is operably linked to the processing enzyme that is operably linked to the sequence SEKDEL for retention in the endoplasmic reticulum. Directing the protein or enzyme to a specific compartment will allow the enzyme to be localized in a manner that it will not come into contact with the substrate. In this manner the enzymatic action of the enzyme will not occur until the enzyme contacts its substrate. The enzyme can be contacted with its substrate by the process of milling (physical disruption of the cell integrity) and hydrating. For example, a mesophilic starch-hydrolyzing enzyme can be targeted to the apoplast or to the endoplasmic reticulum and will therefore not come into contact with starch granules in the amyloplast. Milling of the grain will disrupt the integrity of the grain and the starch hydrolyzing enzyme will then contact the starch granules. In this manner the potential negative effects of co-localization of an enzyme and its substrate can be circumvented.

h. Food Products Without Added Sweetener

Also provided is a method to produce a sweetened farinaceous food product without adding additional sweetener. Examples of farinaceous products include, but are not limited to, breakfast food, ready to eat food, baked food, pasta and cereal products such as

breakfast cereal. The method comprises treating a plant part comprising at least one starch processing enzyme under conditions which activate the starch processing enzyme, thereby processing starch granules in the plant part to sugars so as to form a sweetened product, e.g., relative to the product produced by processing starch granules from a plant part which does not comprise the hyperthermophilic enzyme. Preferably, the starch processing enzyme is hyperthermophilic and is activated by heating, such as by baking, boiling, heating, steaming, electrical discharge, or any combination thereof. The plant part is obtained from a transformed plant, for instance from transformed soybean, rye, oat, barley, wheat, corn, rice or sugar cane, the genome of which is augmented with an expression cassette encoding the at least one hyperthermophilic starch processing enzyme, e.g.,  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase, glucose isomerase, or any combination thereof. The sweetened product is then processed into a farinaceous food product. The invention also provides a farinaceous food product, e.g., a cereal food, a breakfast food, a ready to eat food, or a baked food, produced by the method. The farinaceous food product may be formed from the sweetened product and water, and may contain malt, flavorings, vitamins, minerals, coloring agents or any combination thereof.

The enzyme may be activated to convert polysaccharides contained within the plant material into sugar prior to inclusion of the plant material into the cereal product or during the processing of the cereal product. Accordingly, polysaccharides contained within the plant material may be converted into sugar by activating the material, such as by heating in the case of a hyperthermophilic enzyme, prior to inclusion in the farinaceous product. The plant material containing sugar produced by conversion of the polysaccharides is then added to the product to produce a sweetened product. Alternatively, the polysaccharides may be converted into sugars by the enzyme during the processing of the farinaceous product. Examples of processes used to make cereal products are well known in the art and include heating, baking, boiling and the like as described in U.S. Patent Nos.: 6,183,788; 6,159,530; 6,149,965; 4,988,521 and 5,368,870.

Briefly, dough may be prepared by blending various dry ingredients together with water and cooking to gelatinize the starchy components and to develop a cooked flavor. The cooked material can then be mechanically worked to form a cooked dough, such as cereal dough. The



dry ingredients may include various additives such as sugars, starch, salt, vitamins, minerals, colorings, flavorings, salt and the like. In addition to water, various liquid ingredients such as corn (maize) or malt syrup can be added. The farinaceous material may include cereal grains, cut grains, grits or flours from wheat, rice, corn, oats, barley, rye, or other cereal grains and mixtures thereof from that a transformed plant of the invention. The dough may then be processed into a desired shape through a process such as extrusion or stamping and further cooked using means such as a James cooker, an oven or an electrical discharge device.

Further provided is a method to sweeten a starch containing product without adding sweetener. The method comprises treating starch comprising at least one starch processing enzyme conditions to activate the at least one enzyme thereby digesting the starch to form a sugar thereby forming a treated (sweetened) starch, e.g., relative to the product produced by treating starch which does not comprise the hyperthermophilic enzyme. The starch of the invention is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one processing enzyme. Enzymes include  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase, glucose isomerase, or any combination thereof. The enzyme may be hyperthermophilic and activated with heat. Preferred transformed plants include corn, soybean, rye, oat, barley, wheat, rice and sugar cane. The treated starch is then added to a product to produce a sweetened starch containing product, e.g., a farinaceous food product. Also provided is a sweetened starch containing product produced by the method.

The invention further provides a method to sweeten a polysaccharide containing fruit or vegetable comprising: treating a fruit or vegetable comprising at least one polysaccharide processing enzyme under conditions which activate the at least one enzyme, thereby processing the polysaccharide in the fruit or vegetable to form sugar, yielding a sweetened fruit or vegetable, e.g., relative to a fruit or vegetable from a plant which does not comprise the polysaccharide processing enzyme. The fruit or vegetable of the invention is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one polysaccharide processing enzyme.

Fruits and vegetables include potato, tomato, banana, squash, pea, and bean.

Enzymes include  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase, glucose

isomerase, or any combination thereof. The enzyme may be hyperthermophilic.

i. Sweetening a polysaccharide containing plant or plant product

The method involves obtaining a plant that expresses a polysaccharide processing enzyme which converts a polysaccharide into a sugar as described above. Accordingly the enzyme is expressed in the plant and in the products of the plant, such as in a fruit or vegetable. In one embodiment, the enzyme is placed under the control of an inducible promoter such that expression of the enzyme may be induced by an external stimulus. Such inducible promoters and constructs are well known in the art and are described herein. Expression of the enzyme within the plant or product thereof causes polysaccharide contained within the plant or product thereof to be converted into sugar and to sweeten the plant or product thereof. In another embodiment, the polysaccharide processing enzyme is constitutively expressed. Thus, the plant or product thereof may be activated under conditions sufficient to activate the enzyme to convert the polysaccharides into sugar through the action of the enzyme to sweeten the plant or product thereof. As a result, this self-processing of the polysaccharide in the fruit or vegetable to form sugar yields a sweetened fruit or vegetable, e.g., relative to a fruit or vegetable from a plant which does not comprise the polysaccharide processing enzyme. The fruit or vegetable of the invention is obtained from a transformed plant, the genome of which is augmented with an expression cassette encoding the at least one polysaccharide processing enzyme. Fruits and vegetables include potato, tomato, banana, squash, pea, and bean. Enzymes include  $\alpha$ -amylase,  $\alpha$ -glucosidase, glucoamylase, pullulanase, glucose isomerase, or any combination thereof. The polysaccharide processing enzyme may be hyperthermophilic.

j. Isolation of starch from transformed grain that contains a enzyme which disrupts the endosperm matrix

The invention provides a method to isolate starch from a transformed grain wherein an enzyme is expressed that disrupts the endosperm matrix. The method involves obtaining a plant that expresses an enzyme which disrupts the endosperm matrix by modification of, for example, cell walls, non-starch polysaccharides and/or proteins. Examples of such enzymes include, but are not limited to, proteases, glucanases, thioredoxin, thioredoxin reductase, phytases, lipases, cellulases, beta glucosidases, xylanases and esterases. Such enzymes do not include any enzyme

that exhibits starch-degrading activity so as to maintain the integrity of the starch granules. The enzyme may be fused to a signal sequence that targets the enzyme to the starch granule. In one embodiment the grain is heat dried to activate the enzyme and inactivate the endogenous enzymes contained within the grain. The heat treatment causes activation of the enzyme, which acts to disrupt the endosperm matrix which is then easily separated from the starch granules. In another embodiment, the grain is steeped at low or high temperature, with high or low moisture content, with or without sulfur dioxide. The grain is then heat treated to disrupt the endosperm matrix and allow for easy separation of the starch granules. In another embodiment, proper temperature and moisture conditions are created to allow proteases to enter into the starch granules and degrade proteins contained within the granules. Such treatment would produce starch granules with high yield and little contaminating protein.

k. Syrup having a high sugar equivalent and use of the syrup to produce ethanol or a fermented beverage

The method involves obtaining a plant that expresses a polysaccharide processing enzyme which converts a polysaccharide into a sugar as described above. The plant, or product thereof, is steeped in an aqueous stream under conditions where the expressed enzyme converts polysaccharide contained within the plant, or product thereof, into dextrin, maltooligosaccharide, and/or sugar. The aqueous stream containing the dextrin, maltooligosaccharide, and/or sugar produced through conversion of the polysaccharide is then separated to produce a syrup having a high sugar equivalent. The method may or may not include an additional step of wet-milling the plant or product thereof to obtain starch granules. Examples of enzymes that may be used within the method include, but are not limited to,  $\alpha$ -amylase, glucoamylase, pullulanase and  $\alpha$ -glucosidase. The enzyme may be hyperthermophilic. Sugars produced according to the method include, but are not limited to, hexose, glucose and fructose. Examples of plants that may be used with the method include, but are not limited to, corn, wheat or barley. Examples of products of a plant that may be used include, but are not limited to, fruit, grain and vegetables. In one embodiment, the polysaccharide processing enzyme is placed under the control of an inducible promoter. Accordingly, prior to or during the steeping process, the promoter is induced to cause expression of the enzyme, which then provides for the conversion of

polysaccharide into sugar. Examples of inducible promoters and constructs containing them are well known in the art and are provided herein. Thus, where the polysaccharide processing is hyperthermophilic, the steeping is performed at a high temperature to activate the hyperthermophilic enzyme and inactivate endogenous enzymes found within the plant or product thereof. In another embodiment, a hyperthermophilic enzyme able to convert polysaccharide into sugar is constitutively expressed. This enzyme may or may not be targeted to a compartment within the plant through use of a signal sequence. The plant, or product thereof, is steeped under high temperature conditions to cause the conversion of polysaccharides contained within the plant into sugar.

Also provided is a method to produce ethanol or a fermented beverage from syrup having a high sugar equivalent. The method involves incubating the syrup with yeast under conditions that allow conversion of sugar contained within the syrup into ethanol or a fermented beverage. Examples of such fermented beverages include, but are not limited to, beer and wine. Fermentation conditions are well known in the art and are described in U.S. Patent No.: 4,929,452 and herein. Preferably the yeast is a high alcohol-tolerant and high-sugar tolerant strain of yeast such as *S. cerevisiae* ATCC No. 20867. The fermented product or fermented beverage may be distilled to isolate ethanol or a distilled beverage.

1. **Accumulation of hyperthermophilic enzyme in the cell wall of a plant**

The invention provides a method to accumulate a hyperthermophilic enzyme in the cell wall of a plant. The method involves expressing within a plant a hyperthermophilic enzyme that is fused to a cell wall targeting signal such that the targeted enzyme accumulates in the cell wall. Preferably the enzyme is able to convert polysaccharides into monosaccharides. Examples of targeting sequences include, but are not limited to, a cellulose or xylose binding domain. Examples of hyperthermophilic enzymes include those listed in SEQ ID NO: 1, 3, 5, 10, 13, 14, 15 or 16. Plant material containing cell walls may be added as a source of desired enzymes in a process to recover sugars from the feedstock or as a source of enzymes for the conversion of polysaccharides originating from other sources to monosaccharides. Additionally, the cell walls may serve as a source from which enzymes may be purified. Methods to purify enzymes are well known in the art and include, but are not limited to, gel filtration, ion-exchange chromatography, chromatofocusing, isoelectric focusing, affinity chromatography, FPLC,

HPLC, salt precipitation, dialysis, and the like. Accordingly, the invention also provides purified enzymes isolated from the cell walls of plants.

m. Method of preparing and isolating processing enzymes

In accordance with the present invention, recombinantly-produced processing enzymes of the present invention may be prepared by transforming plant tissue or plant cell comprising the processing enzyme of the present invention capable of being activated in the plant, selected for the transformed plant tissue or cell, growing the transformed plant tissue or cell into a transformed plant, and isolating the processing enzyme from the transformed plant or part thereof. The recombinantly-produced enzyme may be an  $\alpha$ -amylase, glucoamylase, glucose isomerase,  $\alpha$ -glucosidase, pullulanase, xylanase, protease, glucanase, beta glucosidase, esterase, lipase, or phytase. The enzyme may be encoded by the polynucleotide selected from any of SEQ ID NO: 2, 4, 6, 9, 19, 21, 25, 37, 39, 41, 43, 46, 48, 50, 52, 59, 61, 63, 65, 79, 81, 83, 85, 87, 89, 91, 93, 94, 95, 96, 97, or 99.

The invention will be further described by the following examples, which are not intended to limit the scope of the invention in any manner.

### **Examples**

#### **Example 1**

#### **Construction of maize-optimized genes for hyperthermophilic starch-processing/isomerization enzymes**

The enzymes,  $\alpha$ -amylase, pullulanase,  $\alpha$ -glucosidase, and glucose isomerase, involved in starch degradation or glucose isomerization were selected for their desired activity profiles. These include, for example, minimal activity at ambient temperature, high temperature activity/stability, and activity at low pH. The corresponding genes were then designed by using maize preferred codons as described in U.S. Patent No. 5,625,136 and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

The 797GL3  $\alpha$ -amylase, having the amino acid sequence SEQ ID NO:1, was selected for its hyperthermophilic activity. This enzyme's nucleic acid sequence was deduced and maize-



optimized as represented in SEQ ID NO:2. Similarly, the 6gp3 pullulanase was selected having the amino acid sequence set forth in SEQ ID NO:3. The nucleic acid sequence for the 6gp3 pullulanase was deduced and maize-optimized as represented in SEQ ID NO:4.

The amino acid sequence for malA  $\alpha$ -glucosidase from *Sulfolobus solfataricus* was obtained from the literature, J. Bact. 177:482-485 (1995); J. Bact. 180:1287-1295 (1998). Based on the published amino acid sequence of the protein (SEQ ID NO:5), the maize-optimized synthetic gene (SEQ ID NO:6) encoding the malA  $\alpha$ -glucosidase was designed.

Several glucose isomerase enzymes were selected. The amino acid sequence (SEQ ID NO:18) for glucose isomerase derived from *Thermotoga maritima* was predicted based on the published DNA sequence having Accession No. NC\_000853 and a maize-optimized synthetic gene was designed (SEQ ID NO: 19). Similarly the amino acid sequence (SEQ ID NO:20) for glucose isomerase derived from *Thermotoga neapolitana* was predicted based on the published DNA sequence from Appl. Envir. Microbiol. 61(5):1867-1875 (1995), Accession No. L38994. A maize-optimized synthetic gene encoding the *Thermotoga neapolitana* glucose isomerase was designed (SEQ ID NO:21).

## Example 2

### Expression of fusion of 797GL3 $\alpha$ -amylase and starch encapsulating region in *E. coli*

A construct encoding hyperthermophilic 797GL3  $\alpha$ -amylase fused to the starch encapsulating region (SER) from maize granule-bound starch synthase (waxy) was introduced and expressed in *E. coli*. The maize granule-bound starch synthase cDNA (SEQ ID NO:7) encoding the amino acid sequence (SEQ ID NO:8)(Klosgen RB, et al. 1986) was cloned as a source of a starch binding domain, or starch encapsulating region (SER). The full-length cDNA was amplified by RT-PCR from RNA prepared from maize seed using primers SV57 (5'AGCGAATTCATGGCGGCTCTGGCCACGT 3') (SEQ ID NO: 22) and SV58 (5'AGCTAAGCTTCAGGGCGCGGCCACGTTCT 3') (SEQ ID NO: 23) designed from GenBank Accession No. X03935. The complete cDNA was cloned into pBluescript as an EcoRI/HindIII fragment and the plasmid designated pNOV4022.

The C-terminal portion (encoded by bp 919-1818) of the waxy cDNA, including the starch-binding domain, was amplified from pNOV4022 and fused in-frame to the 3' end of the full-length maize-optimized 797GL3 gene (SEQ ID NO:2). The fused gene product, 797GL3/Waxy, having the nucleic acid SEQ ID NO:9 and encoding the amino acid sequence, SEQ ID NO:10, was cloned as an NcoI/XbaI fragment into pET28b (NOVAGEN, Madison, WI) that was cut with NcoI/NheI. The 797GL3 gene alone was also cloned into the pET28b vector as an NcoI/XbaI fragment.

The pET28/797GL3 and the pET28/797GL3/Waxy vectors were transformed into BL21/DE3 *E. coli* cells (NOVAGEN) and grown and induced according to the manufacturer's instruction. Analysis by PAGE/Coomassie staining revealed an induced protein in both extracts corresponding to the predicted sizes of the fused and unfused amylase, respectively.

Total cell extracts were analyzed for hyperthermophilic amylase activity as follows: 5 mg of starch was suspended in 20 µl of water then diluted with 25 µl of ethanol. The standard amylase positive control or the sample to be tested for amylase activity was added to the mixture and water was added to a final reaction volume of 500 µl. The reaction was carried out at 80°C for 15-45 minutes. The reaction was then cooled down to room temperature, and 500 µl of o-dianisidine and glucose oxidase/peroxidase mixture (Sigma) was added. The mixture was incubated at 37°C for 30 minutes. 500 µl of 12 N sulfuric acid was added to stop the reaction. Absorbance at 540 nm was measured to quantitate the amount of glucose released by the amylase/sample. Assay of both the fused and unfused amylase extracts gave similar levels of hyperthermophilic amylase activity, whereas control extracts were negative. This indicated that the 797GL3 amylase was still active (at high temperatures) when fused to the C-terminal portion of the waxy protein.

### Example 3

#### Isolation of promoter fragments for endosperm-specific expression in maize.

The promoter and 5' noncoding region I (including the first intron) from the large subunit of *Zea mays* ADP-gpp (ADP-glucose pyrophosphorylase) was amplified as a 1515 base pair fragment (SEQ ID NO:11) from maize genomic DNA using primers designed from Genbank

accession M81603. The ADP-gpp promoter has been shown to be endosperm-specific (Shaw and Hannah, 1992).

The promoter from the *Zea mays*  $\gamma$ -zein gene was amplified as a 673 bp fragment (SEQ ID NO:12) from plasmid pGZ27.3 (obtained from Dr. Brian Larkins). The  $\gamma$ -zein promoter has been shown to be endosperm-specific (Torrent et al. 1997).

#### Example 4

##### Construction of transformation vectors for the 797GL3 hyperthermophilic $\alpha$ -amylase

Expression cassettes were constructed to express the 797GL3 hyperthermophilic amylase in maize endosperm with various targeting signals as follows:

pNOV6200 (SEQ ID NO:13) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic 797GL3 amylase as described above in Example 1 for targeting to the endoplasmic reticulum and secretion into the apoplast (Torrent et al. 1997). The fusion was cloned behind the maize ADP-gpp promoter for expression specifically in the endosperm.

pNOV6201 (SEQ ID NO:14) comprises the  $\gamma$ -zein N-terminal signal sequence fused to the synthetic 797GL3 amylase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum (ER) (Munro and Pelham, 1987). The fusion was cloned behind the maize ADP-gpp promoter for expression specifically in the endosperm.

pNOV7013 comprises the  $\gamma$ -zein N-terminal signal sequence fused to the synthetic 797GL3 amylase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum (ER). PNOV7013 is the same as pNOV6201, except that the the maize  $\gamma$ -zein promoter (SEQ ID NO:12) was used instead of the maize ADP-spp promoter in order to express the fusion in the endosperm.

pNOV4029 (SEQ ID NO:15) comprises the waxy amyloplast targeting peptide (Klosgen et al., 1986) fused to the synthetic 797GL3 amylase for targeting to the amyloplast. The fusion was cloned behind the maize ADP-gpp promoter for expression specifically in the endosperm.

pNOV4031 (SEQ ID NO:16) comprises the waxy amyloplast targeting peptide fused to the synthetic 797GL3/waxy fusion protein for targeting to starch granules. The fusion was cloned behind the maize ADP-gpp promoter for expression specifically in the endosperm.

Additional constructs were made with these fusions cloned behind the maize  $\gamma$ -zein promoter to obtain higher levels of enzyme expression. All expression cassettes were moved into a binary vector for transformation into maize via *Agrobacterium* infection. The binary vector contained the phosphomannose isomerase (PMI) gene which allows for selection of transgenic cells with mannose. Transformed maize plants were either self-pollinated or outcrossed and seed was collected for analysis.

Additional constructs were made with the targeting signals described above fused to either 6gp3 pullulanase or to 340g12  $\alpha$ -glucosidase in precisely the same manner as described for the  $\alpha$ -amylase. These fusions were cloned behind the maize ADP-gpp promoter and/or the  $\gamma$ -zein promoter and transformed into maize as described above. Transformed maize plants were either self-pollinated or outcrossed and seed was collected for analysis.

Combinations of the enzymes can be produced either by crossing plants expressing the individual enzymes or by cloning several expression cassettes into the same binary vector to enable cotransformation.

### **Example 5**

#### **Construction of plant transformation vectors for the 6GP3 thermophillic pullulanase**

An expression cassette was constructed to express the 6GP3 thermophillic pullulanase in the endoplasmic reticulum of maize endosperm as follows:

pNOV7005 (SEQ ID NOs:24 and 25) comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the synthetic 6GP3 pullulanase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the ER. The amino acid peptide SEKDEL was fused to the C-terminal end of the enzymes using PCR with primers designed to amplify the synthetic gene and simultaneously add the 6 amino acids at the C-terminal end of the protein. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

### **Example 6**

#### **Construction of plant transformation vectors for the malA**

hyperthermophilic  $\alpha$ -glucosidase

Expression cassettes were constructed to express the *Sulfolobus solfataricus* malA hyperthermophilic  $\alpha$ -glucosidase in maize endosperm with various targeting signals as follows:

pNOV4831 (SEQ ID NO:26) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic malA  $\alpha$ -glucosidase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum (ER) (Munro and Pelham, 1987). The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

pNOV4839 (SEQ ID NO:27) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic malA  $\alpha$ -glucosidase for targeting to the endoplasmic reticulum and secretion into the apoplast (Torrent et al. 1997). The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

pNOV4837 comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic malA  $\alpha$ -glucosidase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the ER. The fusion was cloned behind the maize ADPgpp promoter for expression specifically in the endosperm. The amino acid sequence for this clone is identical to that of pNOV4831 (SEQ ID NO:26).



**Example 7****Construction of plant transformation vectors for the hyperthermophilic  
*Thermotoga maritima* and *Thermotoga neapolitana* glucose isomerases**

Expression cassettes were constructed to express the *Thermotoga maritima* and *Thermotoga neapolitana* hyperthermophilic glucose isomerases in maize endosperm with various targeting signals as follows:

pNOV4832 (SEQ ID NO:28) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic *Thermotoga maritima* glucose isomerase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the ER. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

pNOV4833 (SEQ ID NO:29) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic *Thermotoga neapolitana* glucose isomerase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the ER. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

pNOV4840 (SEQ ID NO:30) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic *Thermotoga neapolitana* glucose isomerase for targeting to the endoplasmic reticulum and secretion into the apoplast (Torrent et al. 1997). The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

pNOV4838 comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic *Thermotoga neapolitana* glucose isomerase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the ER. The fusion was cloned behind the maize ADPgpp promoter for expression specifically in the endosperm. The amino acid sequence for this clone is identical to that of pNOV4833 (SEQ ID NO:29).

**Example 8****Construction of plant transformation vectors for the expression of the hyperthermophilic glucanase EglA**

pNOV4800 (SEQ ID NO:58) comprises the barley alpha amylase AMY32b signal sequence (MGKNGNLCCFSLLLLLLAGLASGHQ)(SEQ ID NO:31) fused with the EglA mature protein sequence for localization to the apoplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**Example 9****Construction of plant transformation vectors for the expression of multiple hyperthermophilic enzymes**

pNOV4841 comprises a double gene construct of a 797GL3  $\alpha$ -amylase fusion and a 6GP3 pullulanase fusion. Both 797GL3 fusion (SEQ ID NO:33) and 6GP3 fusion (SEQ ID NO:34) possessed the maize  $\gamma$ -zein N-terminal signal sequence and SEKDEL sequence for targeting to and retention in the ER. Each fusion was cloned behind a separate maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

pNOV4842 comprises a double gene construct of a 797GL3  $\alpha$ -amylase fusion and a malA  $\alpha$ -glucosidase fusion. Both the 797GL3 fusion polypeptide (SEQ ID NO:35) and malA  $\alpha$ -glucosidase fusion polypeptide (SEQ ID NO:36) possess the maize  $\gamma$ -zein N-terminal signal sequence and SEKDEL sequence for targeting to and retention in the ER. Each fusion was cloned behind a separate maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

pNOV4843 comprises a double gene construct of a 797GL3  $\alpha$ -amylase fusion and a malA  $\alpha$ -glucosidase fusion. Both the 797GL3 fusion and malA  $\alpha$ -glucosidase fusion possess the maize  $\gamma$ -zein N-terminal signal sequence and SEKDEL sequence for targeting to and retention in the ER. The 797GL3 fusion was cloned behind the maize  $\gamma$ -zein promoter and the malA fusion was cloned behind the maize ADPgpp promoter for expression specifically in the endosperm. The amino acid sequences of the 797GL3 fusion and the malA fusion are identical to those of pNOV4842 (SEQ ID Nos: 35 and 36, respectively).

pNOV4844 comprises a triple gene construct of a 797GL3  $\alpha$ -amylase fusion, a 6GP3 pullulanase fusion, and a malA  $\alpha$ -glucosidase fusion. 797GL3, malA, and 6GP3 all possess the

maize  $\gamma$ -zein N-terminal signal sequence and SEKDEL sequence for targeting to and retention in the ER. The 797GL3 and malA fusions were cloned behind 2 separate maize  $\gamma$ -zein promoters, and the 6GP3 fusion was cloned behind the maize ADPgpp promoter for expression specifically in the endosperm. The amino acid sequences for the 797GL3 and malA fusions are identical to those of pNOV4842 (SEQ ID Nos: 35 and 36, respectively). The amino acid sequence for the 6GP3 fusion is identical to that of the 6GP3 fusion in pNOV4841 (SEQ ID NO:34).

All expression cassettes set forth in this Example as well as in the Examples that follow were moved into the binary vector pNOV2117 for transformation into maize via *Agrobacterium* infection. pNOV2117 contains the phosphomannose isomerase (PMI) gene allowing for selection of transgenic cells with mannose. pNOV2117 is a binary vector with both the pVS1 and ColE1 origins of replication. This vector contains the constitutive VirG gene from pAD1289 (Hansen, G., et al., PNAS USA 91:7603-7607 (1994), incorporated by reference herein) and a spectinomycin resistance gene from Tn7. Cloned into the polylinker between the right and left borders are the maize ubiquitin promoter, PMI coding region and nopaline synthase terminator of pNOV117 (Negrotto, D., et al., Plant Cell Reports 19:798-803 (2000), incorporated by reference herein). Transformed maize plants will either be self-pollinated or outcrossed and seed collected for analysis. Combinations of the different enzymes can be produced either by crossing plants expressing the individual enzymes or by transforming a plant with one of the multi-gene cassettes.

### Example 10

#### Construction of bacterial and *Pichia* expression vectors

Expression cassettes were constructed to express the hyperthermophilic  $\alpha$ -glucosidase and glucose isomerases in either *Pichia* or bacteria as follows:

pNOV4829 (SEQ ID NOS: 37 and 38) comprises a synthetic *Thermotoga maritima* glucose isomerase fusion with ER retention signal in the bacterial expression vector pET29a. The glucose isomerase fusion gene was cloned into the NcoI and SacI sites of pET29a, which results in the addition of an N-terminal S-tag for protein purification.

pNOV4830 (SEQ ID NOS: 39 and 40) comprises a synthetic *Thermotoga neapolitana* glucose isomerase fusion with ER retention signal in the bacterial expression vector pET29a.

The glucose isomerase fusion gene was cloned into the NcoI and SacI sites of pET29a, which results in the addition of an N-terminal S-tag for protein purification.

pNOV4835 (SEQ ID NO: 41 and 42) comprises the synthetic *Thermotoga maritima* glucose isomerase gene cloned into the BamHI and EcoRI sites of the bacterial expression vector pET28C. This resulted in the fusion of a His-tag (for protein purification) to the N-terminal end of the glucose isomerase.

pNOV4836 (SEQ ID NO: 43 AND 44) comprises the synthetic *Thermotoga neapolitana* glucose isomerase gene cloned into the BamHI and EcoRI sites of the bacterial expression vector pET28C. This resulted in the fusion of a His-tag (for protein purification) to the N-terminal end of the glucose isomerase.

### Example 11

Transformation of immature maize embryos was performed essentially as described in Negrotto et al., Plant Cell Reports 19: 798-803. For this example, all media constituents are as described in Negrotto et al., *supra*. However, various media constituents described in the literature may be substituted.

#### A. Transformation plasmids and selectable marker

The genes used for transformation were cloned into a vector suitable for maize transformation. Vectors used in this example contained the phosphomannose isomerase (PMI) gene for selection of transgenic lines (Negrotto et al. (2000) Plant Cell Reports 19: 798-803).

#### B. Preparation of *Agrobacterium tumefaciens*

*Agrobacterium* strain LBA4404 (pSB1) containing the plant transformation plasmid was grown on YEP (yeast extract (5 g/L), peptone (10g/L), NaCl (5g/L), 15g/l agar, pH 6.8) solid medium for 2 – 4 days at 28°C. Approximately  $0.8 \times 10^9$  *Agrobacterium* were suspended in LS-inf media supplemented with 100  $\mu$ M As (Negrotto *et al.*, (2000) Plant Cell Rep 19: 798-803). Bacteria were pre-induced in this medium for 30-60 minutes.

### C. Inoculation

Immature embryos from A188 or other suitable genotype were excised from 8 – 12 day old ears into liquid LS-inf + 100  $\mu$ M As. Embryos were rinsed once with fresh infection medium. *Agrobacterium* solution was then added and embryos were vortexed for 30 seconds and allowed to settle with the bacteria for 5 minutes. The embryos were then transferred scutellum side up to LSAs medium and cultured in the dark for two to three days. Subsequently, between 20 and 25 embryos per petri plate were transferred to LSDc medium supplemented with cefotaxime (250 mg/l) and silver nitrate (1.6 mg/l) and cultured in the dark for 28°C for 10 days.

### D. Selection of transformed cells and regeneration of transformed plants

Immature embryos producing embryogenic callus were transferred to LSD1M0.5S medium. The cultures were selected on this medium for 6 weeks with a subculture step at 3 weeks. Surviving calli were transferred to Reg1 medium supplemented with mannose. Following culturing in the light (16 hour light/ 8 hour dark regiment), green tissues were then transferred to Reg2 medium without growth regulators and incubated for 1-2 weeks. Plantlets are transferred to Magenta GA-7 boxes (Magenta Corp, Chicago Ill.) containing Reg3 medium and grown in the light. After 2-3 weeks, plants were tested for the presence of the PMI genes and other genes of interest by PCR. Positive plants from the PCR assay were transferred to the greenhouse.

### Example 12

#### Analysis of T1 seed from maize plants expressing the $\alpha$ -amylase targeted to apoplast or to the ER

T1 seed from self-pollinated maize plants transformed with either pNOV6200 or pNOV6201 as described in Example 4 were obtained. Starch accumulation in these kernels appeared to be normal, based on visual inspection and on normal staining for starch with an iodine solution prior to any exposure to high temperature. Immature kernels were dissected and purified endosperms were placed individually in microfuge tubes and immersed in 200  $\mu$ l of 50 mM NaPO<sub>4</sub> buffer. The tubes were placed in an 85°C water bath for 20 minutes, then cooled on ice. Twenty microliters of a 1% iodine solution was added to each tube and mixed. Approximately 25% of the segregating kernels stained normally for starch. The remaining 75% failed to stain, indicating that the starch had been degraded into low molecular weight sugars that



do not stain with iodine. It was found that the T1 kernels of pNOV6200 and pNOV6201 were self-hydrolyzing the corn starch. There was no detectable reduction in starch following incubation at 37°C.

Expression of the amylase was further analyzed by isolation of the hyperthermophilic protein fraction from the endosperm followed by PAGE/Coomassie staining. A segregating protein band of the appropriate molecular weight (50 kD) was observed. These samples are subjected to an  $\alpha$ -amylase assay using commercially available dyed amylose (AMYLASYME, from Megazyme, Ireland). High levels of hyperthermophilic amylase activity correlated with the presence of the 50 kD protein.

It was further found that starch in kernels from a majority of transgenic maize, which express hyperthermophilic  $\alpha$ -amylase, targeted to the amyloplast, is sufficiently active at ambient temperature to hydrolyze most of the starch if the enzyme is allowed to be in direct contact with a starch granule. Of the eighty lines having hyperthermophilic  $\alpha$ -amylase targeted to the amyloplast, four lines were identified that accumulate starch in the kernels. Three of these lines were analyzed for thermostable  $\alpha$ -amylase activity using a colorimetric amylazyme assay (Megazyme). The amylase enzyme assay indicated that these three lines had low levels of thermostable amylase activity. When purified starch from these three lines was treated with appropriate conditions of moisture and heat, the starch was hydrolyzed indicating the presence of adequate levels of  $\alpha$ -amylase to facilitate the auto-hydrolysis of the starch prepared from these lines.

T1 seed from multiple independent lines of both pNOV6200 and pNOV6201 transformants was obtained. Individual kernels from each line were dissected and purified endosperms were homogenized individually in 300  $\mu$ l of 50 mM NaPO<sub>4</sub> buffer. Aliquots of the endosperm suspensions were analyzed for  $\alpha$ -amylase activity at 85°C. Approximately 80% of the lines segregate for hyperthermophilic activity (See Figures 1A, 1B, and 2).

Kernels from wild type plants or plants transformed with pNOV6201 were heated at 100°C for 1, 2, 3, or 6 hours and then stained for starch with an iodine solution. Little or no starch was detected in mature kernels after 3 or 6 hours, respectively. Thus, starch in mature

kernels from transgenic maize which express hyperthermophilic amylase that is targeted to the endoplasmic reticulum was hydrolyzed when incubated at high temperature.

In another experiment, partially purified starch from mature T1 kernels from pNOV6201 plants that were steeped at 50°C for 16 hours was hydrolyzed after heating at 85°C for 5 minutes. This illustrated that the  $\alpha$ -amylase targeted to the endoplasmic reticulum binds to starch after grinding of the kernel, and is able to hydrolyze the starch upon heating. Iodine staining indicated that the starch remains intact in mature seeds after the 16 hour steep at 50°C.

In another experiment, segregating, mature kernels from plants transformed with pNOV6201 were heated at 95°C for 16 hours and then dried. In seeds expressing the hyperthermophilic  $\alpha$ -amylase, the hydrolysis of starch to sugar resulted in a wrinkled appearance following drying.

### **Example 13**

#### Analysis of T1 seed from maize plants expressing the $\alpha$ -amylase targeted to the amyloplast

T1 seed from self-pollinated maize plants transformed with either pNOV4029 or pNOV4031 as described in Example 4 was obtained. Starch accumulation in kernels from these lines was clearly not normal. All lines segregated, with some variation in severity, for a very low or no starch phenotype. Endosperm purified from immature kernels stained only weakly with iodine prior to exposure to high temperatures. After 20 minutes at 85°C, there was no staining. When the ears were dried, the kernels shriveled up. This particular amylase clearly had sufficient activity at greenhouse temperatures to hydrolyze starch if allowed to be in direct contact with the granule

### **Example 14**

#### Fermentation of grain from maize plants expressing $\alpha$ -amylase

100% Transgenic grain 85°C vs. 95°C, varied liquefaction time.

Transgenic corn (pNOV6201) that contains a thermostable  $\alpha$ -amylase performs well in fermentation without addition of exogenous  $\alpha$ -amylase, requires much less time for liquefaction and results in more complete solubilization of starch. Laboratory scale fermentations were

performed by a protocol with the following steps (detailed below): 1) grinding, 2) moisture analysis, 3) preparation of a slurry containing ground corn, water, backset and  $\alpha$ -amylase, 4) liquefaction and 5) simultaneous saccharification and fermentation (SSF). In this example the temperature and time of the liquefaction step were varied as described below. In addition the transgenic corn was liquefied with and without exogenous  $\alpha$ -amylase and the performance in ethanol production compared to control corn treated with commercially available  $\alpha$ -amylase.

The transgenic corn used in this example was made in accordance with the procedures set out in Example 4 using a vector comprising the  $\alpha$ -amylase gene and the PMI selectable marker, namely pNOV6201. The transgenic corn was produced by pollinating a commercial hybrid (N3030BT) with pollen from a transgenic line expressing a high level of thermostable  $\alpha$ -amylase. The corn was dried to 11% moisture and stored at room temperature. The  $\alpha$ -amylase content of the transgenic corn flour was 95 units/g where 1 unit of enzyme generates 1 micromole reducing ends per min from corn flour at 85 °C in pH 6.0 MES buffer. The control corn that was used was a yellow dent corn known to perform well in ethanol production.

1) Grinding: Transgenic corn (1180 g) was ground in a Perten 3100 hammer mill equipped with a 2.0 mm screen thus generating transgenic corn flour. Control corn was ground in the same mill after thoroughly cleaning to prevent contamination by the transgenic corn.

2) Moisture analysis: Samples (20 g) of transgenic and control corn were weighed into aluminum weigh boats and heated at 100 °C for 4 h. The samples were weighed again and the moisture content calculated from the weight loss. The moisture content of transgenic flour was 9.26%; that of the control flour was 12.54%.

3) Preparation of slurries: The composition of slurries was designed to yield a mash with 36% solids at the beginning of SSF. Control samples were prepared in 100 ml plastic bottles and contained 21.50 g of control corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight), and 0.30 ml of a commercially available  $\alpha$ -amylase diluted 1/50 with water. The  $\alpha$ -amylase dose was chosen as representative of industrial usage. When assayed under the conditions described above for assay of the transgenic  $\alpha$ -amylase, the control  $\alpha$ -amylase dose was 2 U/g corn flour. pH was adjusted to 6.0 by addition of ammonium hydroxide. Transgenic samples were prepared in the same fashion but contained 20 g of corn flour because of the lower

moisture content of transgenic flour. Slurries of transgenic flour were prepared either with  $\alpha$ -amylase at the same dose as the control samples or without exogenous  $\alpha$ -amylase.

4) Liquefaction: The bottles containing slurries of transgenic corn flour were immersed in water baths at either 85 °C or 95 °C for times of 5, 15, 30, 45 or 60 min. Control slurries were incubated for 60 min at 85 °C. During the high temperature incubation the slurries were mixed vigorously by hand every 5 min. After the high temperature step the slurries were cooled on ice.

5) Simultaneous saccharification and fermentation: The mash produced by liquefaction was mixed with glucoamylase (0.65 ml of a 1/50 dilution of a commercially available L-400 glucoamylase), protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole was cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash was then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90 F. After 24 hours of fermentation the temperature was lowered to 86 F; at 48 hours it was set to 82 F.

Yeast for inoculation was propagated by preparing a mixture that contained yeast (0.12 g) with 70 grams maltodextrin, 230 ml water, 100 ml backset, glucoamylase (0.88 ml of a 10-fold dilution of a commercially available glucoamylase), protease (1.76 ml of a 100-fold dilution of a commercially available enzyme), urea (1.07 grams), penicillin (0.67 mg) and zinc sulfate (0.13 g). The propagation culture was initiated the day before it was needed and was incubated with mixing at 90°F.

At 24, 48 & 72 hour samples were taken from each fermentation vessel, filtered through 0.2  $\mu$ m filters and analyzed by HPLC for ethanol & sugars. At 72 h samples were analyzed for total dissolved solids and for residual starch.

HPLC analysis was performed on a binary gradient system equipped with refractive index detector, column heater & Bio-Rad Aminex HPX-87H column. The system was equilibrated with 0.005 M H<sub>2</sub>SO<sub>4</sub> in water at 1 ml/min. Column temperature was 50 °C. Sample injection volume was 5  $\mu$ l; elution was in the same solvent. The RI response was calibrated by injection of known standards. Ethanol and glucose were both measured in each injection.

Residual starch was measured as follows. Samples and standards were dried at 50°C in an oven, then ground to a powder in a sample mill. The powder (0.2 g) was weighed into a 15

ml graduated centrifuge tube. The powder was washed 3 times with 10 ml aqueous ethanol (80% v/v) by vortexing followed by centrifugation and discarding of the supernatant. DMSO (2.0 ml) was added to the pellet followed by 3.0 ml of a thermostable alpha-amylase (300 units) in MOPS buffer. After vigorous mixing, the tubes were incubated in a water bath at 85°C for 60 min. During the incubation, the tubes were mixed four times. The samples were cooled and 4.0 ml sodium acetate buffer (200 mM, pH 4.5) was added followed by 0.1 ml of glucoamylase (20 U). Samples were incubated at 50°C for 2 hours, mixed, then centrifuged for 5 min at 3,500 rpm. The supernatant was filtered through a 0.2 um filter and analyzed for glucose by the HPLC method described above. An injection size of 50 µl was used for samples with low residual starch (<20% of solids).

Results Transgenic corn performed well in fermentation without added  $\alpha$ -amylase. The yield of ethanol at 72 hours was essentially the same with or without exogenous  $\alpha$ -amylase as shown in Table I. These data also show that a higher yield of ethanol is achieved when the liquefaction temperature is higher; the present enzyme expressed in the transgenic corn has activity at higher temperatures than other enzymes used commercially such as the *Bacillus liquefaciens*  $\alpha$ -amylase.



Table I

Liquefaction temp °C	Liquefaction time min.	Exogenous $\alpha$ -amylase	# replicates	Mean Ethanol % v/v	Std. Dev. % v/v
85	60	Yes	4	17.53	0.18
85	60	No	4	17.78	0.27
95	60	Yes	2	18.22	ND
95	60	No	2	18.25	ND

When the liquefaction time was varied, it was found that the liquefaction time required for efficient ethanol production was much less than the hour required by the conventional process. Figure 3 shows that the ethanol yield at 72 hours fermentation was almost unchanged from 15 min to 60 min liquefaction. In addition liquefaction at 95°C gave more ethanol at each time point than at the 85°C liquefaction. This observation demonstrates the process improvement achieved by use of a hyperthermophilic enzyme.

The control corn gave a higher final ethanol yield than the transgenic corn, but the control was chosen because it performs very well in fermentation. In contrast the transgenic corn has a genetic background chosen to facilitate transformation. Introducing the  $\alpha$ -amylase-trait into elite corn germplasm by well-known breeding techniques should eliminate this difference.

Examination of the residual starch levels of the beer produced at 72 hours (Figure 4) shows that the transgenic  $\alpha$ -amylase results in significant improvement in making starch available for fermentation; much less starch was left over after fermentation.

Using both ethanol levels and residual starch levels the optimal liquefaction times were 15 min at 95°C and 30 min at 85°C. In the present experiments these times were the total time that the fermentation vessels were in the water bath and thus include a time period during which the temperature of the samples was increasing from room temperature to 85°C or 95°C. Shorter liquefaction times may be optimal in large scale industrial processes that rapidly heat the mash by use of equipment such as jet cookers. Conventional industrial liquefaction processes require holding tanks to allow the mash to be incubated at high temperature for one or more hours. The

present invention eliminates the need for such holding tanks and will increase the productivity of liquefaction equipment.

One important function of  $\alpha$ -amylase in fermentation processes is to reduce the viscosity of the mash. At all time points the samples containing transgenic corn flour were markedly less viscous than the control sample. In addition the transgenic samples did not appear to go through the gelatinous phase observed with all control samples; gelatinization normally occurs when corn slurries are cooked. Thus having the  $\alpha$ -amylase distributed throughout the fragments of the endosperm gives advantageous physical properties to the mash during cooking by preventing formation of large gels that slow diffusion and increase the energy costs of mixing and pumping the mash.

The high dose of  $\alpha$ -amylase in the transgenic corn may also contribute to the favorable properties of the transgenic mash. At 85°C, the  $\alpha$ -amylase activity of the transgenic corn was many times greater activity than the of the dose of exogenous  $\alpha$ -amylase used in controls. The latter was chosen as representative of commercial use rates.

### **Example 15**

#### **Effective function of transgenic corn when mixed with control corn**

Transgenic corn flour was mixed with control corn flour in various levels from 5% to 100% transgenic corn flour. These were treated as described in Example 14. The mashes containing transgenically expressed  $\alpha$ -amylase were liquefied at 85 °C for 30 min or at 95 °C for 15 min; control mashes were prepared as described in Example 14 and were liquefied at 85 °C for 30 or 60 min (one each) or at 95 °C for 15 or 60 min (one each).

The data for ethanol at 48 and 72 hours and for residual starch are given in Table 2. The ethanol levels at 48 hours are graphed in Figure 5; the residual starch determinations are shown in Figure 6. These data show that transgenically expressed thermostable  $\alpha$ -amylase gives very good performance in ethanol production even when the transgenic grain is only a small portion (as low as 5%) of the total grain in the mash. The data also show that residual starch is markedly lower than in control mash when the transgenic grain comprises at least 40% of the total grain.

Table 2

Transgenic grain wt %	85 °C Liquefaction			95 °C Liquefaction		
	Residual Starch	Ethanol	Ethanol	Residual Starch	Ethanol	Ethanol
		48 h	% v/v 72 h		48 h	% v/v 72 h
100	3.58	16.71	18.32	4.19	17.72	21.14
80	4.06	17.04	19.2	3.15	17.42	19.45
60	3.86	17.16	19.67	4.81	17.58	19.57
40	5.14	17.28	19.83	8.69	17.56	19.51
20	8.77	17.11	19.5	11.05	17.71	19.36
10	10.03	18.05	19.76	10.8	17.83	19.28
5	10.67	18.08	19.41	12.44	17.61	19.38
0*	7.79	17.64	20.11	11.23	17.88	19.87

\* Control samples . Values the average of 2 determinations

### Example 16

#### Ethanol production as a function of liquefaction pH using transgenic corn at a rate of 1.5 to 12 % of total corn

Because the transgenic corn performed well at a level of 5-10% of total corn in a fermentation, an additional series of fermentations in which the transgenic corn comprised 1.5 to 12% of the total corn was performed. The pH was varied from 6.4 to 5.2 and the  $\alpha$ -amylase enzyme expressed in the transgenic corn was optimized for activity at lower pH than is conventionally used industrially.

The experiments were performed as described in Example 15 with the following exceptions:

- 1). Transgenic flour was mixed with control flour as a percent of total dry weight at the levels ranging from 1.5% to 12.0%.
- 2). Control corn was N3030BT which is more similar to the transgenic corn than the control used in examples 14 and 15.
- 3). No exogenous  $\alpha$ -amylase was added to samples containing transgenic flour.

4). Samples were adjusted to pH 5.2, 5.6, 6.0 or 6.4 prior to liquefaction. At least 5 samples spanning the range from 0% transgenic corn flour to 12% transgenic corn flour were prepared for each pH.

5). Liquefaction for all samples was performed at 85 °C for 60 min.

The change in ethanol content as a function of fermentation time are shown in Figure 7. This figure shows the data obtained from samples that contained 3% transgenic corn. At the lower pH, the fermentation proceeds more quickly than at pH 6.0 and above; similar behavior was observed in samples with other doses of transgenic grain. The pH profile of activity of the transgenic enzyme combined with the high levels of expression will allow lower pH liquefactions resulting in more rapid fermentations and thus higher throughput than is possible at the conventional pH 6.0 process.

The ethanol yields at 72 hours are shown in Figure 8. As can be seen, on the basis of ethanol yield, the results showed little dependence on the amount of transgenic grain included in the sample. Thus the grain contains abundant amylase to facilitate fermentative production of ethanol. It is also demonstrates that lower pH of liquefaction results in higher ethanol yield.

The viscosity of the samples after liquefaction was monitored and it was observed that at pH 6.0, 6% transgenic grain is sufficient for adequate reduction in viscosity. At pH 5.2 and 5.6, viscosity is equivalent to that of the control at 12% transgenic grain, but not at lower percentages of transgenic grain.

### **Example 17**

#### **Production of fructose from corn flour using thermophilic enzymes**

Corn that expresses the hyperthermophilic  $\alpha$ -amylase, 797GL3, was shown to facilitate production of fructose when mixed with an  $\alpha$ -glucosidase (MalA) and a xylose isomerase (XylA).

Seed from pNOV6201 transgenic plants expressing 797GL3 were ground to a flour in a Kleco cell thus creating amylase flour. Non-transgenic corn kernels were ground in the same manner to generate control flour.

The  $\alpha$ -glucosidase, MalA (from *S. solfataricus*), was expressed in *E. coli*. Harvested bacteria were suspended in 50 mM potassium phosphate buffer pH 7.0 containing 1 mM 4-(2-

aminoethyl)benzenesulfonyl fluoride then lysed in a French pressure cell. The lysate was centrifuged at 23,000 x g for 15 min at 4° C. The supernatant solution was removed, heated to 70° C for 10 min, cooled on ice for 10 min, then centrifuged at 34,000 x g for 30 min at 4° C. The supernatant solution was removed and the MalA concentrated two-fold in centricon 10 devices. The filtrate of the centricon 10 step was retained for use as a negative control for MalA.

Xylose (glucose) isomerase was prepared by expressing the xylA gene of *T. neapolitana* in *E. coli*. Bacteria were suspended in 100 mM sodium phosphate pH 7.0 and lysed by passage through a French pressure cell. After precipitation of cell debris, the extract was heated at 80° C for 10 min then centrifuged. The supernatant solution contained the XylA enzymatic activity. An empty-vector control extract was prepared in parallel with the XylA extract.

Corn flour (60 mg per sample) was mixed with buffer and extracts from *E. coli*. As indicated in Table 3, samples contained amylase corn flour (amylase) or control corn flour (control), 50 µl of either MalA extract (+) or filtrate (-), and 20 µl of either XylA extract (+) or empty vector control (-). All samples also contained 230 µl of 50mM MOPS, 10mM MgSO<sub>4</sub>, and 1 mM CoCl<sub>2</sub>; pH of the buffer was 7.0 at room temperature.

Samples were incubated at 85° C for 18 hours. At the end of the incubation time, samples were diluted with 0.9 ml of 85° C water and centrifuged to remove insoluble material. The supernatant fraction was then filtered through a Centricon3 ultrafiltration device and analyzed by HPLC with ELSD detection.

The gradient HPLC system was equipped with Astec Polymer Amino Column, 5 micron particle size, 250 X 4.6 mm and an Alltech ELSD 2000 detector. The system was pre-equilibrated with a 15:85 mixture of water:acetonitrile. The flow rate was 1 ml/min. The initial conditions were maintained for 5 min after injection followed by a 20 min gradient to 50:50 water:acetonitrile followed by 10 minutes of the same solvent. The system was washed with 20 min of 80:20 water:acetonitrile and then re-equilibrated with the starting solvent. Fructose was eluted at 5.8 min and glucose at 8.7 min.



Table 3

Sample	Corn flour	MalA	XylA	fructose peak area x 10 <sup>-6</sup>	glucose peak area x 10 <sup>-6</sup>
1	amylase	+	+	25.9	110.3
2	amylase	-	+	7.0	12.4
3	amylase	+	-	0.1	147.5
4	amylase	-	-	0	25.9
5	control	+	+	0.8	0.5
6	control	-	+	0.3	0.2
7	control	+	-	1.3	1.7
8	control	-	-	0.2	0.3

The HPLC results also indicated the presence of larger maltooligosaccharides in all samples containing the  $\alpha$ -amylase. These results demonstrate that the three thermophilic enzymes can function together to produce fructose from corn flour at a high temperature.

### Example 18

#### Amylase Flour with Isomerase

In another example, amylase flour was mixed with purified MalA and each of two bacterial xylose isomerases: XylA of *T. maritima*, and an enzyme designated BD8037 obtained from Diversa. Amylase flour was prepared as described in Example 18.

*S. solfataricus* MalA with a 6His purification tag was expressed in *E. coli*. Cell lysate was prepared as described in Example 18, then purified to apparent homogeneity using a nickel affinity resin (Probond, Invitrogen) and following the manufacturer's instructions for native protein purification.

*T. maritima* XylA with the addition of an S tag and an ER retention signal was expressed in *E. coli* and prepared in the same manner as the *T. neapolitana* XylA described in Example 18.

Xylose isomerase BD8037 was obtained as a lyophilized powder and resuspended in 0.4x the original volume of water.

Amylase corn flour was mixed with enzyme solutions plus water or buffer. All reactions contained 60 mg amylase flour and a total of 600 $\mu$ l of liquid. One set of reactions was buffered with 50 mM MOPS, pH 7.0 at room temperature, plus 10mM MgSO<sub>4</sub> and 1 mM CoCl<sub>2</sub>; in a second set of reactions the metal-containing buffer solution was replaced by water. Isomerase enzyme amounts were varied as indicated in Table 4. All reactions were incubated for 2 hours at 90°C. Reaction supernatant fractions were prepared by centrifugation. The pellets were washed with an additional 600 $\mu$ l H<sub>2</sub>O and recentrifuged. The supernatant fractions from each reaction were combined, filtered through a Centricon 10, and analyzed by HPLC with ELSD detection as described in Example 17. The amounts of glucose and fructose observed are graphed in Figure 15.

Table 4

Sample	Amylase flour	Mal A	Isomerase
1	60 mg	+	none
2	60 mg	+	<i>T. maritima</i> , 100 $\mu$ l
3	60 mg	+	<i>T. maritima</i> , 10 $\mu$ l
4	60 mg	+	<i>T. maritima</i> , 2 $\mu$ l
5	60 mg	+	BD8037, 100 $\mu$ l
7	60mg	+	BD8037, 2 $\mu$ l
C	60 mg	none	none

With each of the isomerases, fructose was produced from corn flour in a dose-dependent manner when  $\alpha$ -amylase and  $\alpha$ -glucosidase were present in the reaction. These results demonstrate that the grain-expressed amylase 797GL3 can function with MalA and a variety of different thermophilic isomerases, with or without added metal ions, to produce fructose from corn flour at a high temperature. In the presence of added divalent metal ions, the isomerases can achieve the predicted fructose: glucose equilibrium at 90°C of approximately 55% fructose.

This would be an improvement over the current process using mesophilic isomerases, which requires a chromatographic separation to increase the fructose concentration.

### Example 19

#### Expression of a pullulanase in corn

Transgenic plants that were homozygous for either pNOV7013 or pNOV7005 were crossed to generate transgenic corn seed expressing both the 797GL3  $\alpha$ -amylase and 6GP3 pullulanase.

T1 or T2 seed from self-pollinated maize plants transformed with either pNOV 7005 or pNOV 4093 were obtained. pNOV4093 is a fusion of the maize optimized synthetic gene for 6GP3 (SEQ ID: 3,4) with the amyloplast targeting sequence (SEQ ID NO: 7,8) for localization of the fusion protein to the amyloplast. This fusion protein is under the control of the ADPgpp promoter (SEQ ID NO:11) for expression specifically in the endosperm. The pNOV7005 construct targets the expression of the pullulanase in the endoplasmic reticulum of the endosperm. Localization of this enzyme in the ER allows normal accumulation of the starch in the kernels. Normal staining for starch with an iodine solution was also observed, prior to any exposure to high temperature.

As described in the case of  $\alpha$ -amylase the expression of pullulanase targeted to the amyloplast (pNOV4093) resulted in abnormal starch accumulation in the kernels. When the corn-ears are dried, the kernels shriveled up. Apparently, this thermophilic pullulanase is sufficiently active at low temperatures and hydrolyzes starch if allowed to be in direct contact with the starch granules in the seed endosperm.

Enzyme preparation or extraction of the enzyme from corn-flour: The pullulanase enzyme was extracted from the transgenic seeds by grinding them in Kleco grinder, followed by incubation of the flour in 50mM NaOAc pH 5.5 buffer for 1 hr at RT, with continuous shaking. The incubated mixture was then spun for 15min. at 14000 rpm. The supernatant was used as enzyme source.

Pullulanase assay: The assay reaction was carried out in 96-well plate. The enzyme extracted from the corn flour (100  $\mu$ l) was diluted 10 fold with 900  $\mu$ l of 50mM NaOAc pH5.5 buffer, containing 40 mM  $\text{CaCl}_2$ . The mixture was vortexed, 1 tablet of Limit-Dextrizyme

(azurine-crosslinked-pullulan, from Megazyme) was added to each reaction mixture and incubated at 75 °C for 30 min (or as mentioned). At the end of the incubation the reaction mixtures were spun at 3500 rpm for 15 min. The supernatants were diluted 5 fold and transferred into 96-well flat bottom plate for absorbance measurement at 590 nm. Hydrolysis of azurine-crosslinked-pullulan substrate by the pullulanase produces water-soluble dye fragments and the rate of release of these (measured as the increase in absorbance at 590 nm) is related directly to enzyme activity.

Figure 9 shows the analysis of T2 seeds from different events transformed with pNOV 7005. High expression of pullulanase activity, compared to the non-transgenic control, can be detected in a number of events.

To a measured amount (~100 µg) of dry corn flour from transgenic (expressing pullulanase, or amylase or both the enzymes) and / or control (non-transgenic) 1000 µl of 50 mM NaOAc pH 5.5 buffer containing 40 mM CaCl<sub>2</sub> was added. The reaction mixtures were vortexed and incubated on a shaker for 1 hr. The enzymatic reaction was started by transferring the incubation mixtures to high temperature (75 °C, the optimum reaction temperature for pullulanase or as mentioned in the figures) for a period of time as indicated in the figures. The reactions were stopped by cooling them down on ice. The reaction mixtures were then centrifuged for 10 min. at 14000 rpm. An aliquot (100 µl) of the supernatant was diluted three fold, filtered through 0.2-micron filter for HPLC analysis.

The samples were analyzed by HPLC using the following conditions:

Column: Alltech Prevail Carbohydrate ES 5 micron 250 X 4.6 mm

Detector: Alltech ELSD 2000

Pump: Gilson 322

Injector: Gilson 215 injector/diluter

Solvents: HPLC grade Acetonitrile (Fisher Scientific) and Water (purified by Waters Millipore System)

Gradient used for oligosaccharides of low degree of polymerization (DP 1-15).

Time	%Water	%Acetonitrile
0	15	85
5	15	85
25	50	50
35	50	50
36	80	20
55	80	20
56	15	85
76	15	85

Gradient used for saccharides of high degree of polymerization (DP 20 – 100 and above).

Time	%Water	%Acetonitrile
0	35	65
60	85	15
70	85	15
85	35	65
100	35	65

System used for data analysis: Gilson Unipoint Software System Version 3.2

Figures 10A and 10B show the HPLC analysis of the hydrolytic products generated by expressed pullulanase from starch in the transgenic corn flour. Incubation of the flour of pullulanase expressing corn in reaction buffer at 75 °C for 30 minutes results in production of medium chain oligosaccharides (DP ~10-30) and short amylose chains (DP ~ 100 –200) from cornstarch. This figure also shows the dependence of pullulanase activity on presence of calcium ions.

Transgenic corn expressing pullulanase can be used to produce modified-starch/dextrin that is debranched ( $\alpha$ 1-6 linkages cleaved) and hence will have high level of amylose/straight chain dextrin. Also depending on the kind of starch (*e.g.* waxy, high amylose *etc.*) used the



chain length distribution of the amylose/dextrin generated by the pullulanase will vary, and so will the property of the modified-starch/dextrin.

Hydrolysis of  $\alpha$  1-6 linkage was also demonstrated using pullulan as the substrate. The pullulanase isolated from corn flour efficiently hydrolyzed pullulan. HPLC analysis (as described) of the product generated at the end of incubation showed production of maltotriose, as expected, due to the hydrolysis of the  $\alpha$  1-6 linkages in the pullulan molecules by the enzyme from the corn.

### **Example 20**

#### Expression of pullulanase in corn

Expression of the 6gp3 pullulanase was further analyzed by extraction from corn flour followed by PAGE and Coomassie staining. Corn-flour was made by grinding seeds, for 30 sec., in the Kleco grinder. The enzyme was extracted from about 150mg of flour with 1ml of 50mM NaOAc pH 5.5 buffer. The mixture was vortexed and incubated on a shaker at RT for 1hr, followed by another 15 min incubation at 70 °C. The mixture was then spun down (14000 rpm for 15 min at RT) and the supernatant was used as SDS-PAGE analysis. A protein band of the appropriate molecular weight (95 kDal) was observed. These samples are subjected to a pullulanase assay using commercially available dye-conjugated limit-dextrins (LIMIT-DEXTRIZYME, from Megazyme, Ireland). High levels of thermophilic pullulanase activity correlated with the presence of the 95 kD protein.

The Western blot and ELISA analysis of the transgenic corn seed also demonstrated the expression of ~95 kD protein that reacted with antibody produced against the pullulanase (expressed in *E. coli*).

### **Example 21**

#### Increase in the rate of starch hydrolysis and improved yield of small chain (fermentable) oligosaccharides by the addition of pullulanase expressing corn

The data shown in Figures 11A and 11B was generated from HPLC analysis, as described above, of the starch hydrolysis products from two reaction mixtures. The first reaction indicated as 'Amylase' contains a mixture [1:1 (w/w)] of corn flour samples of  $\alpha$ -amylase expressing transgenic corn made according to the method described in Example 4, for example,

and non-transgenic corn A188; and the second reaction mixture 'Amylase + Pullulanase' contains a mixture [1:1 (w/w)] of corn flour samples of  $\alpha$ -amylase expressing transgenic corn and pullulanase expressing transgenic corn made according to the method described in Example 19. The results obtained support the benefit of use of pullulanase in combination with  $\alpha$ -amylase during the starch hydrolysis processes. The benefits are from the increased rate of starch hydrolysis (Figure 11A) and increase yield of fermentable oligosaccharides with low DP (Figure 11B).

It was found that  $\alpha$ -amylase alone or  $\alpha$ -amylase and pullulanase (or any other combination of starch hydrolytic enzymes) expressed in corn can be used to produce maltodextrin (straight or branched oligosaccharides) (Figures 11A, 11B, 12, and 13A). Depending on the reaction conditions, the type of hydrolytic enzymes and their combinations, and the type of starch used the composition of the maltodextrins produced, and hence their properties, will vary.

Figure 12 depicts the results of an experiment carried out in a similar manner as described for Figure 11. The different temperature and time schemes followed during incubation of the reactions are indicated in the figure. The optimum reaction temperature for pullulanase is 75 °C and for  $\alpha$ -amylase it is >95 °C. Hence, the indicated schemes were followed to provide scope to carry out catalysis by the pullulanase and/or the  $\alpha$ -amylase at their respective optimum reaction temperature. It can be clearly deduced from the result shown that combination of  $\alpha$ -amylase and pullulanase performed better in hydrolyzing cornstarch at the end of 60 min incubation period.

HPLC analysis, as described above (except ~150 mg of corn flour was used in these reactions), of the starch hydrolysis product from two sets of reaction mixtures at the end of 30 min incubation is shown in Figure 13A and 13B. The first set of reactions was incubated at 85 °C and the second one was incubated at 95 °C. For each set there are two reaction mixtures; the first reaction indicated as 'Amylase X Pullulanase' contains flour from transgenic corn (generated by cross pollination) expressing both the  $\alpha$ -amylase and the pullulanase, and the second reaction indicated as 'Amylase' mixture of corn flour samples of  $\alpha$ -amylase expressing transgenic corn and non-transgenic corn A188 in a ratio so as to obtain same amount of  $\alpha$ -amylase activity as is observed in the cross (Amylase X Pullulanase). The total yield of low DP oligosaccharides was

more in case of  $\alpha$ -amylase and pullulanase cross compared to corn expressing  $\alpha$ -amylase alone, when the corn flour samples were incubated at 85 °C. The incubation temperature of 95 °C inactivates (at least partially) the pullulanase enzyme, hence little difference can be observed between 'Amylase X Pullulanase' and 'Amylase'. However, the data for both the incubation temperatures shows significant improvement in the amount of glucose produced (Figure 13B), at the end of the incubation period, when corn flour of  $\alpha$ -amylase and pullulanase cross was used compared to corn expressing  $\alpha$ -amylase alone. Hence use of corn expressing both  $\alpha$ -amylase and pullulanase can be especially beneficial for the processes where complete hydrolysis of starch to glucose is important.

The above examples provide ample support that pullulanase expressed in corn seeds, when used in combination with  $\alpha$ -amylase, improves the starch hydrolysis process. Pullulanase enzyme activity, being  $\alpha$  1-6 linkage specific, debranches starch far more efficiently than  $\alpha$ -amylase (an  $\alpha$  -1-4 linkage specific enzyme) thereby reducing the amount of branched oligosaccharides (*e.g.* limit-dextrin, panose; these are usually non-fermentable) and increasing the amount of straight chain short oligosaccharides (easily fermentable to ethanol *etc.*). Secondly, fragmentation of starch molecules by pullulanase catalyzed debranching increases substrate accessibility for the  $\alpha$ -amylase, hence an increase in the efficiency of the  $\alpha$ -amylase catalyzed reaction results.

### Example 22

To determine whether the 797GL3 alpha amylase and malA alpha-glucosidase could function under similar pH and temperature conditions to generate an increased amount of glucose over that produced by either enzyme alone, approximately 0.35 ug of malA alpha glucosidase enzyme (produced in bacteria) was added to a solution containing 1% starch and starch purified from either non-transgenic corn seed (control) or 797GL3 transgenic corn seed (in 797GL3 corn seed the alpha amylase co-purifies with the starch). In addition, the purified starch from non-transgenic and 797GL3 transgenic corn seed was added to 1% corn starch in the absence of any malA enzyme. The mixtures were incubated at 90°C, pH 6.0 for 1 hour, spun down to remove any insoluble material, and the soluble fraction was analyzed by HPLC for glucose levels. As shown in Figure 14, the 797GL3 alpha-amylase and malA alpha-glucosidase

function at a similar pH and temperature to break down starch into glucose. The amount of glucose generated is significantly higher than that produced by either enzyme alone.

### Example 23

The utility of the *Thermoanaerobacterium* glucoamylase for raw starch hydrolysis was determined. As set forth in Figure 15, the hydrolysis conversion of raw starch was tested with water, barley  $\alpha$ -amylase (commercial preparation from Sigma), *Thermoanaerobacterium* glucoamylase, and combinations thereof were ascertained at room temperature and at 30°C. As shown, the combination of the barley  $\alpha$ -amylase with the *Thermoanaerobacterium* glucoamylase was able to hydrolyze raw starch into glucose. Moreover, the amount of glucose produced by the barley amylase and thermoanaerobacter GA is significantly higher than that produced by either enzyme alone.

### Example 24

#### Maize-optimized genes and sequences for raw-starch hydrolysis and vectors for plant transformation

The enzymes were selected based on their ability to hydrolyze raw-starch at temperatures ranging from approximately 20°-50°C. The corresponding genes or gene fragments were then designed by using maize preferred codons for the construction of synthetic genes as set forth in Example 1.

*Aspergillus shirousami*  $\alpha$ -amylase/glucoamylase fusion polypeptide (without signal sequence) was selected and has the amino acid sequence as set forth in SEQ ID NO: 45 as identified in Biosci. Biotech. Biochem., 56:884-889 (1992); Agric. Biol. Chem. 545:1905-14 (1990); Biosci. Biotechnol. Biochem. 56:174-79 (1992). The maize-optimized nucleic acid was designed and is represented in SEQ ID NO:46.

Similarly, *Thermoanaerobacterium thermosaccharolyticum* glucoamylase was selected, having the amino acid of SEQ ID NO:47 as published in Biosci. Biotech. Biochem., 62:302-308 (1998), was selected. The maize-optimized nucleic acid was designed (SEQ ID NO: 48).

*Rhizopus oryzae* glucoamylase was selected having the amino acid sequence (without signal sequence)(SEQ ID NO: 50), as described in the literature (Agric. Biol. Chem. (1986) 50, pg 957-964). The maize-optimized nucleic acid was designed and is represented in SEQ ID NO:51.

Moreover, the maize  $\alpha$ -amylase was selected and the amino acid sequence (SEQ ID NO: 51) and nucleic acid sequence (SEQ ID NO:52) were obtained from the literature. *See, e.g.*, Plant Physiol. 105:759-760 (1994).

Expression cassettes are constructed to express the *Aspergillus shirousami*  $\alpha$ -amylase/glucoamylase fusion polypeptide from the maize-optimized nucleic acid was designed as represented in SEQ ID NO:46, the *Thermoanaerobacterium thermosaccharolyticum* glucoamylase from the maize-optimized nucleic acid was designed as represented in SEQ ID NO: 48, the *Rhizopus oryzae* glucoamylase was selected having the amino acid sequence (without signal sequence)(SEQ ID NO: 49) from the maize-optimized nucleic acid was designed and is represented in SEQ ID NO:50, and the maize  $\alpha$ -amylase.

A plasmid comprising the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) is fused to the synthetic gene encoding the enzyme. Optionally, the sequence SEKDEL is fused to the C-terminal of the synthetic gene for targeting to and retention in the ER. The fusion is cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm in a plant transformation plasmid. The fusion is delivered to the corn tissue via *Agrobacterium* transfection.

### Example 25

Expression cassettes comprising the selected enzymes are constructed to express the enzymes. A plasmid comprising the sequence for a raw starch binding site is fused to the synthetic gene encoding the enzyme. The raw starch binding site allows the enzyme fusion to bind to non-gelatinized starch. The raw-starch binding site amino acid sequence (SEQ ID NO:53) was determined based on literature, and the nucleic acid sequence was maize-optimized to give SEQ ID NO:54. The maize-optimized nucleic acid sequence is fused to the synthetic gene encoding the enzyme in a plasmid for expression in a plant.



### Example 26

#### Construction of maize-optimized genes and vectors for plant transformation

The genes or gene fragments were designed by using maize preferred codons for the construction of synthetic genes as set forth in Example 1.

*Pyrococcus furiosus* EGLA, hyperthermophilic endoglucanase amino acid sequence (without signal sequence) was selected and has the amino acid sequence as set forth in SEQ ID NO: 55, as identified in Journal of Bacteriology (1999) 181, pg 284-290.) The maize-optimized nucleic acid was designed and is represented in SEQ ID NO:56.

*Thermus flavus* xylose isomerase was selected and has the amino acid sequence as set forth in SEQ ID NO:57, as described in Applied Biochemistry and Biotechnology 62:15-27 (1997).

Expression cassettes are constructed to express the *Pyrococcus furiosus* EGLA (endoglucanase) from the maize-optimized nucleic acid (SEQ ID NO:56) and the *Thermus flavus* xylose isomerase from a maize-optimized nucleic acid encoding amino acid sequence SEQ ID NO:57. A plasmid comprising the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) is fused to the synthetic maize-optimized gene encoding the enzyme. Optionally, the sequence SEKDEL is fused to the C-terminal of the synthetic gene for targeting to and retention in the ER. The fusion is cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm in a plant transformation plasmid. The fusion is delivered to the corn tissue via *Agrobacterium* transfection.

### Example 27

#### Production of glucose from corn flour using thermophilic enzymes expressed in corn

Expression of the hyperthermophilic  $\alpha$ -amylase, 797GL3 and  $\alpha$ -glucosidase (MalA) were shown to result in production of glucose when mixed with an aqueous solution and incubated at 90 °C

A transgenic corn line (line 168A10B, pNOV4831) expressing MalA enzyme was identified by measuring  $\alpha$ -glucosidase activity as indicated by hydrolysis of p-nitrophenyl- $\alpha$ -glucoside.

Corn kernels from transgenic plants expressing 797GL3 were ground to a flour in a Kleco cell thus creating amylase flour. Corn kernels from transgenic plants expressing MalA were ground to a flour in a Kleco cell thus creating MalA flour. Non-transgenic corn kernels were ground in the same manner to generate control flour.

Buffer was 50 mM MES buffer pH 6.0.

Corn flour hydrolysis reactions: Samples were prepared as indicated in Table 5 below. Corn flour (about 60 mg per sample) was mixed with 40 ml of 50 mM MES buffer, pH 6.0. Samples were incubated in a water bath set at 90°C for 2.5 and 14 hours. At the indicated incubation times, samples were removed and analyzed for glucose content.

The samples were assayed for glucose by a glucose oxidase / horse radish peroxidase based assay. GOPOD reagent contained: 0.2 mg/ml o-dianisidine, 100 mM Tris pH 7.5, 100 U/ml glucose oxidase & 10 U/ml horse radish peroxidase. 20 µl of sample or diluted sample were arrayed in a 96 well plate along with glucose standards (which varied from 0 to 0.22 mg/ml). 100 µl of GOPOD reagent was added to each well with mixing and the plate incubated at 37 °C for 30 min. 100 µl of sulfuric acid (9M) was added and absorbance at 540 nm was read. The glucose concentration of the samples was determined by reference to the standard curve. The quantity of glucose observed in each sample is indicated in Table 5.

Table 5

Sample	WT flour mg	amylase flour mg	MalA flour Mg	Buffer ml	Glucose 2.5 h mg	Glucose 14 h mg
1	66	0	0	40	0	0
2	31	30	0	40	0.26	0.50
3	30	0	31.5	40	0	0.09
4	0	32.2	30.0	40	2.29	12.30
5	0	6.1	56.2	40	1.16	8.52

These data demonstrate that when expression of hyperthermophilic  $\alpha$ -amylase and  $\alpha$ -glucosidase in corn result in a corn product that will generate glucose when hydrated and heated under appropriate conditions.

### Example 28

#### Production of Maltodextrins

Grain expressing thermophilic  $\alpha$ -amylase was used to prepare maltodextrins. The exemplified process does not require prior isolation of the starch nor does it require addition of exogenous enzymes.

Corn kernels from transgenic plants expressing 797GL3 were ground to a flour in a Kleco cell to create "amylase flour". A mixture of 10% transgenic/90% non-transgenic kernels was ground in the same manner to create "10% amylase flour."

Amylase flour and 10% amylase flour (approximately 60 mg/sample) were mixed with water at a rate of 5  $\mu$ l of water per mg of flour. The resulting slurries were incubated at 90°C for up to 20 hours as indicated in Table 6. Reactions were stopped by addition of 0.9 ml of 50 mM EDTA at 85°C and mixed by pipetting. Samples of 0.2 ml of slurry were removed, centrifuged to remove insoluble material and diluted 3x in water.

The samples were analyzed by HPLC with ELSD detection for sugars and maltodextrins. The gradient HPLC system was equipped with Astec Polymer Amino Column, 5 micron particle size,

250 X 4.6 mm and an Alltech ELSD 2000 detector. The system was pre-equilibrated with a 15:85 mixture of water:acetonitrile. The flow rate was 1 ml/min. The initial conditions were maintained for 5 min after injection followed by a 20 min gradient to 50:50 water:acetonitrile followed by 10 minutes of the same solvent. The system was washed with 20 min of 80:20 water:acetonitrile and then re-equilibrated with the starting solvent.

The resulting peak areas were normalized for volume and weight of flour. The response factor of ELSD per  $\mu\text{g}$  of carbohydrate decreases with increasing DP, thus the higher DP maltodextrins represent a higher percentage of the total than indicated by peak area.

The relative peak areas of the products of reactions with 100% amylase flour are shown in Figure 17. The relative peak areas of the products of reactions with 10% amylase flour are shown in Figure 18.

These data demonstrate that a variety of maltodextrin mixtures can be produced by varying the time of heating. The level of  $\alpha$ -amylase activity can be varied by mixing transgenic  $\alpha$ -amylase-expressing corn with wild-type corn to alter the maltodextrin profile.

The products of the hydrolysis reactions described in this example can be concentrated and purified for food and other applications by use of a variety of well defined methods including: centrifugation, filtration, ion-exchange, gel permeation, ultrafiltration, nanofiltration, reverse osmosis, decolorizing with carbon particles, spray drying and other standard techniques known to the art.

### **Example 29**

#### **Effect of time and temperature on maltodextrin production**

The composition of the maltodextrin products of autohydrolysis of grain containing thermophilic  $\alpha$ -amylase may be altered by varying the time and temperature of the reaction.

In another experiment, amylase flour was produced as described in Example 28 above and mixed with water at a ratio of 300 $\mu\text{l}$  water per 60 mg flour. Samples were incubated at 70°, 80°, 90°, or 100° C for up to 90 minutes. Reactions were stopped by addition of 900ml of 50mM EDTA at 90°C, centrifuged to remove insoluble material and filtered through 0.45 $\mu\text{m}$  nylon filters. Filtrates were analyzed by HPLC as described in Example 28.

The result of this analysis is presented in Figure 19. The DP number nomenclature refers to the degree of polymerization. DP2 is maltose; DP3 is maltotriose, etc. Larger DP maltodextrins eluted in a single peak near the end of the elution and are labeled ">DP12". This aggregate includes dextrans that passed through 0.45  $\mu\text{m}$  filters and through the guard column and does not include any very large starch fragments trapped by the filter or guard column.

This experiment demonstrates that the maltodextrin composition of the product can be altered by varying both temperature and incubation time to obtain the desired maltooligosaccharide or maltodextrin product.

### **Example 30**

#### **Maltodextrin production**

The composition of maltodextrin products from transgenic maize containing thermophilic  $\alpha$ -amylase can also be altered by the addition of other enzymes such as  $\alpha$ -glucosidase and xylose isomerase as well as by including salts in the aqueous flour mixture prior to treating with heat.

In another, amylase flour, prepared as described above, was mixed with purified MalA and/or a bacterial xylose isomerase, designated BD8037. *S. sulfotaricus* MalA with a 6His purification tag was expressed in *E. coli*. Cell lysate was prepared as described in Example 28, then purified to apparent homogeneity using a nickel affinity resin (Probond, Invitrogen) and following the manufacturer's instructions for native protein purification. Xylose isomerase BD8037 was obtained as a lyophilized powder from Diversa and resuspended in 0.4x the original volume of water.

Amylase corn flour was mixed with enzyme solutions plus water or buffer. All reactions contained 60 mg amylase flour and a total of 600 $\mu\text{l}$  of liquid. One set of reactions was buffered with 50 mM MOPS, pH 7.0 at room temperature, plus 10mM  $\text{MgSO}_4$  and 1 mM  $\text{CoCl}_2$ ; in a second set of reactions the metal-containing buffer solution was replaced by water. All reactions were incubated for 2 hours at 90°C. Reaction supernatant fractions were prepared by centrifugation. The pellets were washed with an additional 600 $\mu\text{l}$   $\text{H}_2\text{O}$  and re-centrifuged. The supernatant fractions from each reaction were combined, filtered through a Centricon 10, and analyzed by HPLC with ELSD detection as described above.



The results are graphed in Figure 20. They demonstrate that the grain-expressed amylase 797GL3 can function with other thermophilic enzymes, with or without added metal ions, to produce a variety of maltodextrin mixtures from corn flour at a high temperature. In particular, the inclusion of a glucoamylase or  $\alpha$ -glucosidase may result in a product with more glucose and other low DP products. Inclusion of an enzyme with glucose isomerase activity results in a product that has fructose and thus would be sweeter than that produced by amylase alone or amylase with  $\alpha$ -glucosidase. In addition the data indicate that the proportion of DP5, DP6 and DP7 maltooligosaccharides can be increased by including divalent cationic salts, such as  $\text{CoCl}_2$  and  $\text{MgSO}_4$ .

Other means of altering the maltodextrin composition produced by a reaction such as that described here include: varying the reaction pH, varying the starch type in the transgenic or non-transgenic grain, varying the solids ratio, or by addition of organic solvents.

### **Example 31**

#### Preparing dextrans, or sugars from grain without mechanical disruption of the grain prior to recovery of starch-derived products

Sugars and maltodextrans were prepared by contacting the transgenic grain expressing the  $\alpha$ -amylase, 797GL3, with water and heating to 90°C overnight (>14 hours). Then the liquid was separated from the grain by filtration. The liquid product was analyzed by HPLC by the method described in Example 15. Table 6 presents the profile of products detected.

Table 6

Molecular species	Concentration of Products $\mu\text{g} / 25 \mu\text{l}$ injection
Fructose	0.4
Glucose	18.0
Maltose	56.0
DP3*	26.0
DP4*	15.9
DP5*	11.3
DP6*	5.3
DP7*	1.5

\* Quantification of DP3 includes maltotriose and may include isomers of maltotriose that have an  $\alpha(1 \rightarrow 6)$  bond in place of an  $\alpha(1 \rightarrow 4)$  bond. Similarly DP4 to DP7 quantification includes the linear maltooligosaccarides of a given chain length as well as isomers that have one or more  $\alpha(1 \rightarrow 6)$  bonds in place of one or more  $\alpha(1 \rightarrow 4)$  bonds

These data demonstrate that sugars and maltodextrins can be prepared by contacting intact  $\alpha$ -amylase-expressing grain with water and heating. The products can then be separated from the intact grain by filtration or centrifugation or by gravitational settling.

### Example 32

#### Fermentation of raw starch in corn expressing *Rhizopus oryzae* glucoamylase.

Transgenic corn kernels are harvested from transgenic plants made as described in Example 29. The kernels are ground to a flour. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Rhizopus oryzae* (Sequence ID NO: 49) targeted to the endoplasmic reticulum.

The corn kernels are ground to a flour as described in Example 15. Then a mash is prepared containing s 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following

components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86°C; at 48 hours it is set to 82 °C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### Example 33

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The kernels are ground to a flour. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Rhizopus oryzae* (Sequence ID NO: 49) targeted to the endoplasmic reticulum.

The corn kernels are ground to a flour as described in Example 15. Then a mash is prepared containing 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### Example 34

Example of fermentation of raw starch in whole kernels of corn expressing  
*Rhizopus oryzae* glucoamylase with addition of exogenous  $\alpha$ -amylase

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Rhizopus oryzae* (Sequence ID NO: 49) targeted to the endoplasmic reticulum.

The corn kernels are contacted with 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added: barley  $\alpha$ -amylase purchased from Sigma (2 mg), protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mixture in order to allow CO<sub>2</sub> to vent. The mixture is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### Example 35

#### Fermentation of raw starch in corn expressing *Rhizopus oryzae* glucoamylase and *Zea mays* amylase

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Rhizopus oryzae* (Sequence ID NO:49) targeted to the endoplasmic reticulum. The kernels also express the maize amylase with raw starch binding domain as described in Example 28.

The corn kernels are ground to a flour as described in Example 14. Then a mash is prepared containing 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The

mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90 F. After 24 hours of fermentation the temperature is lowered to 86 F; at 48 hours it is set to 82 F.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### Example 36

#### Example of fermentation of raw starch in corn expressing

#### *Thermoanaerobacter thermosaccharolyticum* glucoamylase.

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Thermoanaerobacter thermosaccharolyticum* (Sequence ID NO: 47) targeted to the endoplasmic reticulum.

The corn kernels are ground to a flour as described in Example 15. Then a mash is prepared containing 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### Example 37

#### Example of fermentation of raw starch in corn expressing

#### *Aspergillus niger* glucoamylase

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Aspergillus niger* (Fiil,N.P. "Glucoamylases G1 and G2 from *Aspergillus niger*



are synthesized from two different but closely related mRNAs" EMBO J. 3 (5), 1097-1102 (1984), Accession number P04064). The maize-optimized nucleic acid encoding the glucoamylase has SEQ ID NO:59 and is targeted to the endoplasmic reticulum.

The corn kernels are ground to a flour as described in Example 14. Then a mash is prepared containing 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### Example 38

#### Example of fermentation of raw starch in corn expressing

#### *Aspergillus niger* glucoamylase and *Zea mays* amylase

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Aspergillus niger* (Fiil,N.P. "Glucoamylases G1 and G2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs" EMBO J. 3 (5), 1097-1102 (1984) : Accession number P04064)(SEQ ID NO:59, maize-optimized nucleic acid) and is targeted to the endoplasmic reticulum. The kernels also express the maize amylase with raw starch binding domain as described in example 28.

The corn kernels are ground to a flour as described in Example 14. Then a mash is prepared containing 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor).

A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### Example 39

#### Example of fermentation of raw starch in corn expressing

##### *Thermoanaerobacter thermosaccharolyticum* glucoamylase and barley amylase

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Thermoanaerobacter thermosaccharolyticum* (Sequence ID NO: 47) targeted to the endoplasmic reticulum. The kernels also express the low pI barley amylase amy1 gene (Rogers, J.C. and Milliman, C. "Isolation and sequence analysis of a barley alpha-amylase cDNA clone" J. Biol. Chem. 258 (13), 8169-8174 (1983) modified to target expression of the protein to the endoplasmic reticulum.

The corn kernels are ground to a flour as described in Example 14. Then a mash is prepared containing 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

**Example 40****Example of fermentation of raw starch in whole kernels of corn expressing *Thermoanaerobacter thermosaccharolyticum* glucoamylase and barley amylase.**

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a protein that contains an active fragment of the glucoamylase of *Thermoanaerobacter thermosaccharolyticum* (Sequence ID NO: 47) targeted to the endoplasmic reticulum. The kernels also express the low pI barley amylase *amyl* gene (Rogers, J.C. and Milliman, C. "Isolation and sequence analysis of a barley alpha-amylase cDNA clone" J. Biol. Chem. 258 (13), 8169-8174 (1983) modified to target expression of the protein to the endoplasmic reticulum.

The corn kernels are contacted with 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mixture: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mixture is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

**Example 41****Example of fermentation of raw starch in corn expressing an alpha-amylase and glucoamylase fusion.**

Transgenic corn kernels are harvested from transgenic plants made as described in Example 28. The corn kernels express a maize-optimized polynucleotide such as provided in SEQ ID NO: 46, encoding an alpha-amylase and glucoamylase fusion, such as provided in SEQ ID NO: 45, which are targeted to the endoplasmic reticulum. .

The corn kernels are ground to a flour as described in Example 14. Then a mash is prepared containing 20 g of corn flour, 23 ml of de-ionized water, 6.0 ml of backset (8% solids

by weight). pH is adjusted to 6.0 by addition of ammonium hydroxide. The following components are added to the mash: protease (0.60 ml of a 1,000-fold dilution of a commercially available protease), 0.2 mg Lactocide & urea (0.85 ml of a 10-fold dilution of 50% Urea Liquor). A hole is cut into the cap of the 100 ml bottle containing the mash to allow CO<sub>2</sub> to vent. The mash is then inoculated with yeast (1.44 ml) and incubated in a water bath set at 90° C. After 24 hours of fermentation the temperature is lowered to 86° C; at 48 hours it is set to 82° C.

Yeast for inoculation is propagated as described in Example 14.

Samples are removed as described in example 14 and then analyzed by the methods described in Example 14.

### **Example 42**

#### Construction of transformation vectors

Expression cassettes were constructed to express the hyperthermophilic beta-glucanase EglA in maize as follows:

**pNOV4800** comprises the barley Amy32b signal peptide (MGKNGNLCCFSLLLLLLAGLASGHQ) fused to the synthetic gene for the EglA beta-glucanase for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**pNOV4803** comprises the barley Amy32b signal peptide fused to the synthetic gene for the EglA beta-glucanase for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize ubiquitin promoter for expression throughout the plant.

Expression cassettes were constructed to express the thermophilic beta-glucanase/mannanase 6GP1 (SEQ ID NO: 85) in maize as follows:

**pNOV4819** comprises the tobacco PR1a signal peptide (MGFVLFSQLPSFLLVSTLLLFLVISHSCRA) fused to the synthetic gene for the 6GP1 beta-glucanase/mannanase for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**pNOV4820** comprises the synthetic gene for 6GP1 cloned behind the maize  $\gamma$ -zein promoter for cytoplasmic localization and expression specifically in the endosperm.

**pNOV4823** comprises the tobacco PR1a signal peptide fused to the synthetic gene for the 6GP1 beta-glucanase/mannanase with a C-terminal addition of the sequence KDEL for targeting to and retention in the endoplasmic reticulum. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**pNOV4825** comprises the tobacco PR1a signal peptide fused to the synthetic gene for the 6GP1 beta-glucanase/mannanase with a C-terminal addition of the sequence KDEL for targeting to and retention in the endoplasmic reticulum. The fusion was cloned behind the maize ubiquitin promoter for expression throughout the plant.

Expression cassettes were constructed to express the barley AmyI alpha-amylase (SEQ ID NO: 87) in maize as follows:

**pNOV4867** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the barley AmyI alpha-amylase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**pNOV4879** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the barley AmyI alpha-amylase with a C-terminal addition of the sequence SEKDEL for targeting to and retention



in the endoplasmic reticulum. The fusion was cloned behind the maize globulin promoter for expression specifically in the embryo.

**pNOV4897** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the barley AmyI alpha-amylase for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize globulin promoter for expression specifically in the embryo.

**pNOV4895** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the barley AmyI alpha-amylase for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm

**pNOV4901** comprises the gene for the barley AmyI alpha-amylase cloned behind the maize globulin promoter for cytoplasmic localization and expression specifically in the embryo.

Expression cassettes were constructed to express the *Rhizopus* glucoamylase (SEQ ID NO: 50) in maize as follows:

**pNOV4872** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the synthetic gene for *Rhizopus* glucoamylase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**pNOV4880** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the synthetic gene for *Rhizopus* glucoamylase with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum. The fusion was cloned behind the maize globulin promoter for expression specifically in the embryo.

**pNOV4889** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the synthetic gene for *Rhizopus* glucoamylase for targeting to the endoplasmic reticulum and secretion into the

apoplast. The fusion was cloned behind the maize globulin promoter for expression specifically in the embryo.

**pNOV4890** comprises the maize  $\gamma$ -zein N-terminal signal sequence fused to the synthetic gene for *Rhizopus* glucoamylase for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**pNOV4891** comprises the synthetic gene for *Rhizopus* glucoamylase cloned behind the maize  $\gamma$ -zein promoter for cytoplasmic localization and expression specifically in the endosperm.

### **Example 43**

#### Expression of the mesophilic *Rhizopus* glucoamylase in corn

A variety of constructs were generated for the expression of the *Rhizopus* glucoamylase in corn. The maize  $\gamma$ -zein and globulin promoters were used to express the glucoamylase specifically in the endosperm or embryo, respectively. In addition, the maize  $\gamma$ -zein signal sequence and a synthetic ER retention signal were used to regulate the subcellular localization of the glucoamylase protein. All 5 constructs (pNOV4872, pNOV4880, pNOV4889, pNOV4890, and pNOV4891) yielded transgenic plants with glucoamylase activity detected in the seed. Tables 7 and 8 show the results for individual transgenic seed (construct pNOV4872) and pooled seed (construct pNOV4889), respectively. No detrimental phenotype was observed for any transgenic plants expressing this *Rhizopus* glucoamylase.

**Glucoamylase assay:** Seed were ground to a flour and the flour was suspended in water. The samples were incubated at 30 degrees for 50 minutes to allow the glucoamylase to react with the starch. The insoluble material was pelleted and the glucose concentration was determined for the supernatants. The amount of glucose liberated in each sample was taken as an indication of the level of glucoamylase present. Glucose concentration was determined by incubating the samples with GOHOD reagent (300mM Tris/Cl pH7.5, glucose oxidase

(20U/ml), horseradish peroxidase (20U/ml), o-dianisidine 0.1 mg/ml) for 30 minutes at 37 degrees C, adding 0.5 volumes of 12N H<sub>2</sub>SO<sub>4</sub>, and measuring the OD<sub>540</sub>.

Table 7 shows activity of the *Rhizopus* glucoamylase in individual transgenic corn seed (construct pNOV4872).

Table 7

<b>Seed</b>	<b>U/g flour</b>
Wild Type #1	0.07
Wild Type #2	0.55
Wild Type #3	0.25
Wild Type #4	0.33
Wild Type #5	0.30
Wild Type #6	0.42
Wild Type #7	-0.01
Wild Type #8	0.31
MD9L022156 #1	5.17
MD9L022156 #2	1.66
MD9L022156 #3	7.66
MD9L022156 #4	1.77
MD9L022156 #5	7.08
MD9L022156 #6	4.46
MD9L022156 #7	2.20
MD9L022156 #8	3.50
MD9L023377 #1	9.23
MD9L023377 #2	4.30
MD9L023377 #3	6.72
MD9L023377 #4	3.35
MD9L023377 #5	0.56
MD9L023377 #6	4.79
MD9L023377 #7	4.60
MD9L023377 #8	6.01
MD9L023043 #1	4.93
MD9L023043 #2	8.74
MD9L023043 #3	2.70
MD9L023043 #4	0.72
MD9L023043 #5	3.33
MD9L023043 #6	3.53
MD9L023043 #7	3.94
MD9L023043 #8	11.51

MD9L023334 #1	4.28
MD9L023334 #2	2.86
MD9L023334 #3	0.56
MD9L023334 #4	6.96
MD9L023334 #5	3.29
MD9L023334 #6	3.18
MD9L023334 #7	4.57
MD9L023334 #8	7.44
MD9L022039 #1	6.25
MD9L022039 #2	2.85
MD9L022039 #3	4.32
MD9L022039 #4	2.51
MD9L022039 #5	5.06
MD9L022039 #6	5.03
MD9L022039 #7	2.79
MD9L022039 #8	2.98

Table 8 shows activity of the *Rhizopus* glucoamylase in pooled transgenic corn seed (construct pNOV4889).

**Table 8**

<b>Seed</b>	<b>U/g flour</b>
Wild Type	0.38
MD9L023347	2.14
MD9L023352	2.34
MD9L023369	1.66
MD9L023469	1.42
MD9L023477	1.33
MD9L023482	1.95
MD9L023484	1.32
MD9L024170	1.35
MD9L024177	1.48
MD9L024184	1.60
MD9L024186	1.34
MD9L024196	1.38
MD9L024228	1.69
MD9L024263	1.70
MD9L024315	1.32

MD9L024325	1.73
MD9L024333	1.41
MD9L024339	1.84

All expression cassettes were inserted into the binary vector pNOV2117 for transformation into maize via *Agrobacterium* infection. The binary vector contained the phosphomannose isomerase (PMI) gene which allows for selection of transgenic cells with mannose. Transformed maize plants were either self-pollinated or outcrossed and seed was collected for analysis.

#### Example 44

##### Expression of the hyperthermophilic beta-glucanase EglA in corn

For expression of the hyperthermophilic beta-glucanase EglA in corn we utilized the ubiquitin promoter for expression throughout the plant and the  $\gamma$ -zein promoter for expression specifically in the endosperm of corn seed. The barley Amy32b signal peptide was fused to EglA for localization in the apoplast.

Expression of the hyperthermophilic beta-glucanase EglA in transgenic corn seed and leaves was analysed using an enzymatic assay and western blotting.

Transgenic seed segregating for construct pNOV4800 or pNOV4803 were analysed using both western blotting and an enzymatic assay for beta-glucanase. Endosperm was isolated from individual seed after soaking in water for 48 hours. Protein was extracted by grinding the endosperm in 50mM NaPO<sub>4</sub> buffer (pH 6.0). Heat-stable proteins were isolated by heating the extracts at 100 degrees C for 15 minutes, followed by pelleting of the insoluble material. The supernatant containing heat-stable proteins was analysed for beta glucanase activity using the azo-barley glucan method (megazyme). Samples were pre-incubated at 100 degrees C for 10 minutes and assayed for 10 minutes at 100 degrees C using the azo-barley glucan substrate. Following incubation, 3 volumes of precipitation solution were added to each sample, the samples were centrifuged for 1 minute, and the OD590 of each supernatant was determined. In addition, 5ug of protein were separated by SDS-PAGE and blotted to nitrocellulose for western



blot analysis using antibodies against the EglA protein. Western blot analysis detected a specific, heat-stable protein(s) in the EglA positive endosperm extracts, and not in negative extracts. The western blot signal correlates with the level of EglA activity detected enzymatically.

EglA activity was analysed in leaves and seed of plants containing the transgenic constructs pNOV4803 and pNOV4800, respectively. The assays (conducted as described above) showed that the heat-stable beta-glucanase EglA was expressed at various levels in the leaves (Table 9) and seed (Table 10) of transgenic plants while no activity was detected in non-transgenic control plants. Expression of EglA in corn utilizing constructs pNOV4800 and pNOV4803 did not result in any detectable negative phenotype.

Table 9 shows the activity of the hyperthermophilic beta-glucanase EglA in leaves of transgenic corn plants. Enzymatic assays were conducted on extracts from leaves of pNOV4803 transgenic plants to detect hyperthermophilic beta-glucanase activity. Assays were conducted at 100 degrees C using the azo-barley glucan method (megazyme). The results indicate that the transgenic leaves have varying levels of hyperthermophilic beta-glucanase activity.

Table 9

<b>Plant</b>	<b>Abs590</b>
Wild Type	0
266A-17D	0.008
266A-18E	0.184
266A-13C	0.067
266A-15E	0.003
266A-11E	0
265C-1B	0.024
265C-1C	0.065
265C-2D	0.145
265C-5C	0.755
265C-5D	0.133
265C-3A	0.076
266A-4B	0.045
266A-12B	0.066
266A-11C	0.096

266A-14B	0.074
266A-4C	0.107
266A-4A	0.084
266A-12A	0.054
266A-15B	0.052
266A-11A	0.109
266A-20C	0.044
266A-19D	0.02
266A-12C	0.098
266A-4E	0.248
266A-18B	0.367
265C-3D	0.066
266A-20E	0.163
266A-13D	0.084
265C-3B	0.065
266A-15A	0.131
266A-13A	0.169
265C-3E	0.116
266A-20A	0.365
266A-20B	0.521
266A-19C	0.641
266A-20D	0.561
266A-4D	0.363
266A-18A	0.676
265C-5E	0.339
266A-17E	0.221
266A-11B	0.251
265C-4E	0.138
265C-4D	0.242

Table 10 shows the activity of the hyperthermophilic beta-glucanase EglA in seed of transgenic corn plants. Enzymatic assays were conducted on extracts from individual, segregating seed of pNOV4800 transgenic plants to detect hyperthermophilic beta-glucanase activity. Assays were conducted at 100 degrees C using the azo-barley glucan method (megazyme). The results indicate that the transgenic seed have varying levels of hyperthermophilic beta-glucanase activity.

**Table 10**

<b>Seed</b>	<b>Abs 590</b>
Wild Type	0
1A	1.1
1B	0
1C	1.124
1D	1.323
2A	0
2B	1.354
2C	1.307
2D	0
3A	0.276
3B	0.089
3C	0.463
3D	0
4A	0.026
4B	0.605
4C	0.599
4D	0.642
5A	1.152
5B	1.359
5C	1.035
5D	0
6A	0.006
6B	1.201
6C	0.034
6D	1.227
7A	0.465
7B	0
7C	0.366
7D	0.77
8A	1.494
8B	1.427

8C	0.003
8D	1.413

## Effect of transgenic expression of endoglucanase EglA on cell wall composition & in vitro digestibility analysis

Five individual seed from each of two lines, #263 & #266, not expressing or expressing EglA (pNOV4803) respectively were grown in the greenhouse. Protein extracts made from small leaf samples from immature plants were used to verify that transgenic endoglucanase activity was present in #266 plants but not #263 plants. At full plant maturity, ~30 days after pollination, the whole above ground plant was harvested, roughly chopped, and oven dried for 72 hours. Each sample was divided into 2 duplicate samples (labelled A & B respectively), and subjected to in vitro digestibility analysis using strained rumen fluid using common procedures (Forage fiber analysis apparatus, reagents, procedures, and some applications, by H. K. Goering and P. J. Van Soest, Goering, H. Keith 1941 (Washington, D.C.) : Agricultural Research Service, U.S. Dept. of Agriculture, 1970. iv, 20 p. : ill. -- Agriculture handbook ; no. 379 ), except that material was treated by a pre-incubation at either 40°C or 90°C prior to in vitro digestibility analysis. In vitro digestibility analysis was performed as follows:

Samples were chopped to about 1mm with a wiley mill, and then sub-divided into 16 weighed aliquots for analysis. Material was suspended in buffer and incubated at either 40°C or 90°C for 2 hours, then cooled overnight. Micronutrients, trypticase & casein & sodium sulfite were added, followed by strained rumen fluid, and incubated for 30 hours at 37°C. Analyses of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (AD-L) were performed using standard gravimetric methods (Van Soest & Wine, Use of Detergents in the Analysis of fibrous Feeds. IV. Determination of plant cell-wall constituents. P.J. Van Soest & R.H. Wine. (1967). Journal of The AOAC, 50: 50-55; see also Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition (1991). P.J. Van Soest, J.B. Robertson & B.A. Lewis. J. Dairy Science, 74: 3583-3597.).

Data show that transgenic plants expressing EglA (#266) contain more NDF than control plants (#233), whilst ADF & lignin are relatively unchanged. The NDF fraction of transgenic



plants is more readily digested than that of non-transgenic plants, and this is due to an increase in the digestibility of cellulose (NDF – ADF – AD-L), consistent with “self-digestion” of the cell-wall cellulose by the transgenically expressed endoglucanase enzyme.

### Example 45

#### Expression of the thermophilic beta-glucanase/mannanase (6GP1) in corn

Transgenic seed for pNOV4820 and pNOV4823 were analysed for 6GP1 beta glucanase activity using the azo-barley glucan method (megazyme). Enzymatic assays conducted at 50 degrees C indicate that the transgenic seed have thermophilic 6GP1 beta-glucanase activity while no activity was detected in non-transgenic seed (positive signal represents background noise associated with this assay).

Table 11 shows activity of the thermophilic beta-glucanase/mannanase 6GP1 in transgenic corn seed. Transgenic seed for pNOV4820 (events 1-6) and pNOV4823 (events 7-9) were analysed for 6GP1 beta-glucanase activity using the azo-barley glucan method (megazyme). Enzymatic assays were conducted at 50 degrees C and the results indicate that the transgenic seed have thermophilic 6GP1 beta-glucanase activity while no activity is detected in non-transgenic seed.

Table 11

Seed	Abs 590
Wild Type	0
1	0.21
2	0.31
3	0.36
4	0.23
5	0.16
6	0.14
7	0.52
8	0.54
9	0.49

### Example 46

Expression of the mesophilic barley AmyI amylase in corn

A variety of constructs were generated for the expression of the barley AmyI alpha-amylase in corn. The maize  $\gamma$ -zein and globulin promoters were used to express the amylase specifically in the endosperm or embryo, respectively. In addition, the maize  $\gamma$ -zein signal sequence and a synthetic ER retention signal were used to regulate the subcellular localization of the amylase protein. All 5 constructs (pNOV4867, pNOV4879, pNOV4897, pNOV4895, pNOV4901) yielded transgenic plants with alpha-amylase activity detected in the seed. Table 12 shows the activity in individual seed for 5 independent, segregating events (constructs pNOV4879 and pNOV4897). All of the constructs produced some transgenic events with a shrivelled seed phenotype indicating that synthesis of the barley AmyI amylase could effect starch formation, accumulation, or breakdown.

Table 12 shows activity of the barley AmyI alpha-amylase in individual corn seed (constructs pNOV4879 and pNOV4897). Individual, segregating seed for constructs pNOV4879 (seed samples 1 and 2) and pNOV4897 (seed samples 3-5) were analysed for alpha-amylase activity as described previously.

Table 12

Seed	U/g corn flour
1A	19.29
1B	1.49
1C	18.36
1D	1.15
1E	1.62
1F	14.99
1G	1.88
1H	1.83
2A	2.05
2B	36.79

2C	30.11
2D	2.25
2E	32.37
2F	1.92
2G	20.24
2H	35.76
3A	22.99
3B	1.72
3C	25.38
3D	18.41
3E	28.51
3F	2.11
3G	16.67
3H	1.89
4A	1.57
4B	36.14
4C	23.35
4D	1.70
4E	1.94
4F	14.38
4G	2.09
4H	1.83
5A	11.64
5B	18.20
5C	1.87
5D	2.07
5E	1.71
5F	1.92
5G	12.94
5H	15.25

**Example 47**

### Preparation of Xylanase Constructs

Table 13 lists 9 binary vectors that each contain a unique xylanase expression cassette. The xylanase expression cassettes include a promoter, a synthetic xylanase gene (coding sequence), an intron (PEPC, inverted), and a terminator (35S).

Two synthetic maize-optimized endo-xylanase genes were cloned into binary vector pNOV2117. These two xylanase genes were designated BD7436 (SEQ ID NO: 61) and BD6002A (SEQ ID NO:63). Additional binary vectors containing a third maize-optimized sequence, BD6002B (SEQ ID NO:65) can be made.

Two promoters were used: the maize glutelin-2 promoter (27-kD gamma-zein promoter (SEQ ID NO: 12 ) and the rice glutelin-1 (Osgt1) promoter (SEQ ID NO: 67). The first 6 vectors listed in Table 1 have been used to generate transgenic plants. The last 3 vectors can also be made and used to generate transgenic plants.

Vector 11560 and 11562 encode the polypeptide shown in SEQ ID NO: 62 (BD7436). Constructs 11559 and 11561 encode a polypeptide consisting of SEQ ID NO: 17 fused to the N-terminus of SEQ ID NO: 62. SEQ ID NO: 17 is the 19 amino acid signal sequence from the 27-kD gamma-zein protein.

Vector 12175 encodes the polypeptide shown in SEQ ID NO: 64(BD6002A). Vector 12174 encodes a fusion protein consisting of the gamma-zein signal sequence (SEQ ID NO: 17) fused to the N-terminus of SEQ ID NO: 64.

Vectors pWIN062 and pWIN064 encode the polypeptide shown in SEQ ID NO: 66(BD6002B). Vector pWIN058 encodes a fusion protein consisting of the chloroplast transit peptide of maize waxy protein (SEQ ID NO:68) fused to the N-terminus of SEQ ID NO: 66 .

Table 13 Xylanase binary vectors

Vector	Promoter	Signal Sequence Source	Xylanase Gene
11559	27kD Gamma-zein	27kD Gamma-zein	BD7436
11560	27kD Gamma-zein	None	BD7436
11561	OsGt1	27kD Gamma-zein	BD7436
11562	OsGt1	None	BD7436
12174	27kD Gamma-zein	27kD Gamma-zein	BD6002A
12175	27kD Gamma-zein	None	BD6002A
PWIN058	27kD Gamma-zein	Maize waxy protein	BD6002B
PWIN062	OsGt1	None	BD6002B
PWIN064	27kD Gamma-zein	None	BD6002B

All constructs include an expression cassette for PMI, to allow positive selection of regenerated transgenic tissue on mannose-containing media.

### Example 48

#### Xylanase Activity Assay Results

The data shown in Tables 14 and 15 demonstrate that xylanase activity accumulates in T1 generation seed harvested from regenerated (T0) maize plants stably transformed with binary vectors containing xylanase genes BD7436 (SEQ ID NO: 61 in Example 47) and BD6002A (SEQ ID NO:63 in Example 47). Using an Azo-WAXY assay (Megazyme), activity was detected in extracts from both pooled (segregating) transgenic seed and single transgenic seed.

T1 seed were pulverized and soluble proteins were extracted from flour samples using citrate-phosphate buffer (pH 5.4). Flour suspensions were stirred at room temperature for 60 minutes, and insoluble material was removed by centrifugation. The xylanase activity of the supernatant fraction was measured using the Azo-WAXY assay (McCleary, B.V. "Problems in the measurement of beta-xylanase, beta-glucanase and alpha-amylase in feed enzymes and animal feeds". In proceedings of Second European Symposium on Feed Enzymes" (W.van Hartingsveldt, M. Hessing, J.P. van der Jugt, and W.A.C Somers Eds.), Noordwijkerhout, Netherlands, 25-27 October, 1995). Extracts and substrate were pre-incubated at 37°C. To 1 volume of 1X extract supernatant, 1 volume of substrate (1% Azo-Wheat Arabinoxylan S-AWAXP) was added and then incubated at 37°C for 5 minutes. Xylanase activity in the corn



flour extract depolymerizes the Azo-Wheat Arabinoxylan by an endo-mechanism and produces low molecular weight dyed fragments in the form of xylo-oligomers. After the 5 minute incubation, the reaction was terminated by the addition of 5 volumes of 95% EtOH. Addition of alcohol causes the non-depolymerized dyed substrate to precipitate so that only the lower molecular weight xylo-oligomers remain in solution. Insoluble material was removed by centrifugation. The absorbance of the supernatant fraction was measured at 590nm, and the units of xylanase per gram of flour were determined by comparison to the absorbance values from identical assays using a xylanase standard of known activity. The activity of this standard was determined by a BCA assay. The enzyme activity of the standard was determined using wheat arabinoxylan as substrate and measuring the release of reducing ends by reaction of the reducing ends with 2,2'-bicinchoninic acid (BCA). The substrate was prepared as a 1.4% w/w solution of wheat arabinoxylan (Megazyme P-WAXYM) in 100 mM sodium acetate buffer pH5.30 containing 0.02% sodium azide. The BCA reagent was prepared by combining 50 parts reagent A with 1 part reagent B (reagents A and B were from Pierce, product numbers 23223 and 23224, respectively). These reagents were combined no more than four hours before use. The assay was performed by combining 200 microliters of substrate to 80 microliters of enzyme sample. After incubation at the desired temperature for the desired length of time, 2.80 milliliters of BCA reagent was added. The contents were mixed and placed at 80°C for 30-45 minutes. The contents were allowed to cool and then transferred to cuvettes and the absorbance at 560nm was measured relative to known concentrations of xylose. The choice of enzyme dilution, incubation time, and incubation temperature could be varied by one skilled in the art.

The experimental results shown in Table 14 demonstrate the presence of recombinant xylanase activity in flour prepared from T1 generation corn seed. Seed from 12 T0 plants (derived from independent T-DNA integration events) were analyzed. The 12 transgenic events were derived from 6 different vectors as indicated (refer to Table 13 in Example 47 for description of vectors). Extracts of non-transgenic (negative control) corn flour do not contain measurable xylanase activity (see Table 15). The xylanase activity in these 12 samples ranged from 10-87 units/gram of flour.

Table 14. Analysis of pooled T1 seed.

Vector	Sample	Xylanase Units / Gram of Flour
11559	MD9L013800	63
11559	MD9L012428	58
11560	MD9L011296	33
11560	MD9L011322	21
11561	MD9L012413	87
11561	MD9L012443	83
11562	MD9L012890	13
11562	MD9L013788	12
12174	MD9L022080	16
12174	MD9L022195	10
12175	MD9L022061	74
12175	MD9L022134	69

The results in Table 15 demonstrate the presence of xylanase activity in corn flour derived from single kernels. T1 seed from two T0 plants containing vectors 11561 and 11559 were analyzed. These vectors are described in Example 47. Eight seed from each of the two plants were pulverized and flour samples from each seed were extracted. The table shows results of single assays of each extract. No xylanase activity was found in assays of extracts of seeds 1, 5, and 8 for both transgenic events. These seed represent null segregants. Seed 2, 3, 4, 6, and 7 for both transgenic events accumulated measurable xylanase activity attributable to expression of the recombinant BD7436 gene. All 10 seed that tested positive for xylanase activity ( $>10$  unit/gram flour) had an obvious shriveled or shrunken appearance. By contrast the 6 seed that tested negative for xylanase activity ( $\leq 1$  unit/gram flour) had a normal appearance. This result suggests that the recombinant xylanase depolymerized endogenous (arabino)xylan substrate during seed development and/or maturation.

Table 15. Analysis of single T1 seed.

Vector 11561	Vector 11559
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Seed Number	Xylanase Units / Gram of Flour	Seed Number	Xylanase Units / Gram of Flour
1	0	1	1
2	45	2	52
3	38	3	21
4	40	4	13
5	0	5	0
6	40	6	28
7	32	7	23
8	0	8	0

### Example 49

#### Enhanced starch recovery from corn seed using enzymes

Corn wet-milling includes the steps of steeping the corn kernel, grinding the corn kernel, and separating the components of the kernel. A bench top assay (the Cracked Corn Assay) was developed to mimic the corn wet-milling process

The "Cracked Corn Assay" was used for identifying enzymes that enhance starch yield from maize seed resulting in an improved efficiency of the corn wet milling process. Enzyme delivery was either by exogenous addition, transgenic corn seed, or a combination of both. In addition to the use of enzymes to facilitate separation of the corn components, elimination of SO<sub>2</sub> from the process is also shown.

#### Cracked Corn Assay.

One gram of seed was steeped overnight in 4000, 2000, 1000, 500, 400, 40, or 0 ppm SO<sub>2</sub> at 50 degrees C or 37 degrees C. Seeds were cut in half and the germ removed. Each half seed was cut in half again. Steep water from each steeped seed sample was retained and diluted to a final concentrations ranging from 400 ppm to 0 ppm SO<sub>2</sub>. Two milliliters of the steep water with or without enzymes was added to the de-germed seeds and the samples placed at 50

degrees C or 37degrees C for 2-3 hours. Each enzyme was added at 10 units per sample. All samples were vortexed approximately every 15 minutes. After 2-3 hours the samples were filtered through mira cloth into a 50ml centrifuge tube. The seeds were washed with 2 ml of water and the sample pooled with the first supernatant. The samples were centrifuged for 15 minutes at 3000 rpm. Following centrifugation, the supernatant was poured off and the pellet placed at 37 degrees C to dry. All pellet weights were recorded. Starch and protein determinations were also carried out on samples for determining the starch:protein ratios released during the treatments (data not shown).

#### Analysis of T1 and T2 seed from maize plants expressing 6GP1 endoglucanase in Cracked corn Assay

Transgenic corn (pNOV4819 and pNOV4823) containing a thermostable endoglucanase performed well when analyzed in the Cracked Corn Assay. Recovery of starch from the pNOV4819 line was found to be 2 fold higher in seeds expressing the endoglucanase when steeped in 2000 ppm SO<sub>2</sub>. Addition of a protease and cellobiohydrolase to the endoglucanase seed increased the starch recovery approximately 7 fold over control seeds. See Table 16.

Table 16. Crack Corn Assay results for cytosolic expressed Endoglucanase (pNOV4820). Control line, A188/HiII PNOV4819 lines, 42C6A-1-21 and 27.

Maize Line	Treatment	Starch Pellet Wt. (mg)
A188/HiII Control	No Enzyme	28.4
A188/HiII Control	Bromelain/C8546 10U	109.3
42C6A-1-21	No Enzyme	52.6
42C6A-1-21	Bromelain/C8546 10U	170.4
42C6A-1-27	No Enzyme	60.5
42C6A-1-27	Bromelain/C8546 10U	207.5

Similar results were seen in transgenic seed containing endoglucanase targeted to the ER of the

endosperm (pNOV4823), again resulting in a 2 –7 fold increase in starch recovery when compared to control seed. See Table 17.

Table 17. Crack Corn Assay results for ER expressed endoglucanase (pNOV4823). Control line, A188/Hill; PNOV4823 line, 101D11A-1-28.

Line	Treatment	Starch Pellet Wt (mg)	Starch Pellet Wt (mg)	Mean Wt.
A188/Hill	No Enzyme	22.5	19.1	20.8
101D11A-1-28	No Enzyme	41.2	32	36.6
A188/Hill	10U Bromelian/C8546	78.6	73.8	76.2
101D11A-1-28	10U Bromelian/C8546	169.8	132.6	151.2

These results confirm that expression of an endoglucanase enhances the separation of starch and protein components of the corn seed. Further more it could be shown that reduction or removal of SO<sub>2</sub> during the steeping process resulted in starch recovery that was comparable to or better than normally steeped control seeds. See Table 18. Removal of high levels of SO<sub>2</sub> from the wet-milling process can provide value-added benefits.

Table 18. Comparison of various concentrations of SO<sub>2</sub> on starch recovery from transgenic 6GP1 seed.

Line	Treatment	Starch Pellet Wt (mg)
A188 Control	2000 ppm SO <sub>2</sub>	18.5
JHAF Control	2000 ppm SO <sub>2</sub>	29.1
42C (pNOV4820)	2000 ppm SO <sub>2</sub>	29.5
101C (pNOV4823)	2000 ppm SO <sub>2</sub>	73.1
101D (pNOV4823)	2000 ppm SO <sub>2</sub>	42.5
136A (pNOV4825)	2000 ppm SO <sub>2</sub>	36.6



137A (pNOV4825)	2000 ppm SO <sub>2</sub>	38.6
42C (pNOV4820)	400 ppm SO <sub>2</sub>	18.5
101C (pNOV4823)	400 ppm SO <sub>2</sub>	20.4
101D (pNOV4823)	400 ppm SO <sub>2</sub>	39.7
136A (pNOV4825)	400 ppm SO <sub>2</sub>	26
137A (pNOV4825)	400 ppm SO <sub>2</sub>	26.9
42C (pNOV4820)	0 ppm SO <sub>2</sub>	21.9
101C (pNOV4823)	0 ppm SO <sub>2</sub>	32.5
101D (pNOV4823)	0 ppm SO <sub>2</sub>	39
136A (pNOV4825)	0 ppm SO <sub>2</sub>	17.8
137A (pNOV4825)	0 ppm SO <sub>2</sub>	29.2

### Example 50

#### Construction of transformation vectors for maize optimized bromelain

Expression cassettes were constructed to express the maize optimized bromelain in maize endosperm with various targeting signals as follows:

pSYN11000 (SEQ ID NO. 73 ) comprises the bromelain signal sequence (MAWKVQVVFLFLFLCVMWASPSAASA) (SEQ ID NO: 72) and synthetic bromelain sequence fused with a C-terminal addition of the sequence VFAEAIAANSTLVAE for targeting to and retention in the PVS (Vitale and Raikhel Trends in Plant Science Vol 4 no.4 pg 149-155). The fusion was cloned behind the maize gamma zein promoter for expression specifically in the endosperm.

pSYN11587 (SEQ ID NO:75) comprises the bromelain N-terminal signal sequence (MAWKVQVVFLFLFLCVMWASPSAASA) and synthetic bromelain sequence with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum (ER) (Munro and Pelham, 1987). The fusion was cloned behind the maize gamma zein promoter for expression specifically in the endosperm.

pSYN11589 (SEQ ID NO. 74) comprises the bromelain signal sequence (MAWKVQVVFLFLFLCVMWASPSAASA) (SEQ ID NO: 72) fused to the lytic vacuolar

targeting sequence SSSSFADSNPIRVTDRRAAST (Neuhaus and Rogers Plant Molecular Biology 38:127-144, 1998) and synthetic bromelain for targeting to the lytic vacuole. The fusion was cloned behind the maize gamma zein promoter for expression specifically in the endosperm.

pSYN12169 (SEQ ID NO: 76) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic bromelain for targeting to the endoplasmic reticulum and secretion into the apoplast (Torrent et al. 1997). The fusion was cloned behind the maize gamma zein promoter for expression specifically in the endosperm.

pSYN12575 (SEQ ID NO:77) comprises the waxy amyloplast targeting peptide (Klosgen et al., 1986) fused to the synthetic bromelain for targeting to the amyloplast. The fusion was cloned behind the gamma zein promoter for expression specifically in the endosperm.

pSM270 ( SEQ ID NO.78 ) comprises the bromelain N-terminal signal sequence fused to the lytic vacuolar targeting sequence SSSSFADSNPIRVTDRRAAST (Neuhaus and Rogers Plant Molecular Biology 38:127-144, 1998) and synthetic bromelain for targeting to the lytic vacuole. The fusion was cloned behind the aleurone specific promoter P19 (US Patent 6392123) for expression specifically in the aleurone.

### **Example 51**

#### **Expression of bromelain in corn**

Seeds from T1 transgenic lines transformed with vectors containing the synthetic bromelain gene with targeting sequences for expression in various subcellular location of the seed were analyzed for protease activity. Corn-flour was made by grinding seeds, for 30 sec., in the Kleco grinder. The enzyme was extracted from 100 mg of flour with 1 ml of 50 mM NaOAc pH4.8 or 50 mM Tris pH 7.0 buffer containing 1mM EDTA and 5 mM DTT. Samples were vortexed, then placed at 4C with continuous shaking for 30 min. Extracts from each transgenic line was assayed using resorufin labeled casein (Roche, Cat. No. 1 080 733) as outlined in the product brochure. Flour from T2 seeds were assayed using a bromelain specific assay as outlined in Methods in Enzymology Vol. 244: Pg 557-558 with the following modifications. 100mg of corn seed flour was extracted with 1ml of 50mMNa<sub>2</sub>HPO<sub>4</sub>/50mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 1

mM EDTA +/- 1  $\mu$ M leupeptin for 15 min at 4°C. Extracts were centrifuged for 5 min at 14,000 rpm at 4°C. Extracts were done in duplicates. Flour from T2 Transgenic lines was assayed for bromelain activity using Z-Arg-Arg-NHMec (Sigma) as a substrate. Four aliquots of 100  $\mu$ l /corn seed extracts were added to 96 well flat bottom plates (Corning) containing 50  $\mu$ l 100mM Na<sub>2</sub>HPO<sub>4</sub> /100mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 2mM EDTA, 8mM DTT/well. The reaction was started by the addition of 50  $\mu$ l of 20  $\mu$ M Z-Arg-Arg-NHMec. The reaction rate was monitored using a SpectraFluorPlus (Tecan) fitted with a 360nm excitation and 465nm emission filters at 40°C at 2.5min intervals.

Table 19 shows the analysis of seed from different T1 bromelain events. Bromelain expression was found to be 2-7 fold higher than the A188 and JHAF control lines. T1 transgenic lines were replanted and T2 seeds obtained. Analysis of T2 seeds showed expression of bromelain. Figure 21 shows bromelain activity assay using Z-Arg-Arg-NHMec in T2 seed for ER targeted (11587) and lytic vacuolar targeted (11589) bromelain.

*Analysis of T2 seed from maize plants expressing Bromelain*

Seed from T2 transgenic bromelain line, 11587-2 was analyzed in the Cracked Corn assay for enhanced starch recovery. Previous experiments using exogenously added bromelain showed an increased starch recovery when tested alone and in combination with other enzymes, particularly cellulases. The T2 seed from line 11587-2 showed a 1.3 fold increase in starch recovered over control seed when steeped at 37°C/2000 ppm SO<sub>2</sub> overnight. More importantly, there was the 2 fold increase in starch from the T2 bromelain line, 11587-2 when a cellulase (C8546) was added when seeds were steeped at 37°C/2000 ppm SO<sub>2</sub>.

The transgenic line showed a similar trend in increased starch over control seed when seeds were steeped at 37°C/400 ppm SO<sub>2</sub>. A 1.6 fold increase starch recovered over control was

seen in the transgenic seed and a 2.1 fold increase of starch with addition of a cellulase (C8546).

See Table 20.

These results are significant in showing that it is possible to reduced temperature and SO<sub>2</sub> levels while also enhancing the starch recovery during the wet-milling process when transgenic seed expressing a bromelain is used.

Table 19

Summary of Grain Specific Expression of Bromelain in T1 corn.

Line Number	Targeting	Construct	"Specific Activity" ng Bromelain/protein
11000-1	Vacuolar	GZP/probromelain/barleyPVS	252
11000-2	Vacuolar	GZP/probromelain/barleyPVS	277
11000-3	Vacuolar	GZP/probromelain/barleyPVS	284
11587-1	ER	GZP/probromelain/KDEL	174
11587-1	ER	GZP/probromelain/KDEL	153
11589-1	Lytic Vacuolar	GZP/aleurainSS/probromelain	547
11589-2	Lytic Vacuolar	GZP/aleurainSS/probromelain	223
		A188 Control	56
		JHAF Control	75

Table 20 Cracked Corn Assay results for T2 Bromelain seed

Steep Conditions	Line	Starch Pellet Wt. (mg)
2000 ppm SO <sub>2</sub>	A188	41.3
2000 ppm SO <sub>2</sub>	A188/C8546 (10 units)	44
2000 ppm SO <sub>2</sub>	11587-2	57.4
2000 ppm SO <sub>2</sub>	11587-2/C8546 (10 units)	94.6
400 ppm	A188	30.7
400 ppm	A188/C8546 (10 units)	35.8
400 ppm	11587-2	50.5
400 ppm	11587-2/C8546 (10 units)	86.6

### Example 52

#### Construction of transformation vectors for maize optimized ferulic acid esterase.

Expression cassettes were constructed to express the maize optimize ferulic acid esterase in maize endosperm with or without various targeting signals as follows:

Plasmid 13036 (SEQ ID NO: 101) comprises the maize optimize ferulic acid esterase (FAE) sequence (SEQ ID NO: 99). The sequence was cloned behind the maize gamma zein promoter without any targeting sequences for expression specifically in the cytosol of the endosperm.

Plasmid 13038 (SEQ ID NO: 103) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic FAE for targeting to the endoplasmic reticulum and secretion into the apoplast (Torrent et al. 1997). The fusion was cloned behind the maize gamma zein promoter for expression specifically in the endosperm.

Plasmid 13039 (SEQ ID NO: 105) comprises the waxy amyloplast targeting peptide (MLAALATSQLVATRAGLGVPDASTFRRGAAQGLRGARASAAAD TLSMRTSARAAPRHQHQQARRGARFPSLVVCASAGA) (Klosgen et al., 1986) fused to the synthetic FAE for targeting to the amyloplast. The fusion was cloned behind the gamma zein promoter for expression specifically in the endosperm.

Plasmid 13347 (SEQ ID NO: 107) comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to the synthetic FAE sequence with a C-terminal addition of the sequence SEKDEL for targeting to and retention in the endoplasmic reticulum (ER) (Munro and Pelham, 1987). The fusion was cloned behind the maize gamma zein promoter for expression specifically in the endosperm.

All expression cassettes were moved into a binary vector pNOV2117 for transformation into maize via *Agrobacterium* infection. The binary vector contained the phosphomannose isomerase (PMI) gene which allows for selection of transgenic cells with mannose. Transformed maize plants were either self-pollinated or outcrossed and seed was collected for analysis.



Combinations of the enzymes can be produced either by crossing plants expressing the individual enzymes or by cloning several expression cassettes into the same binary vector to enable cotransformation.

Synthetic Ferulic Acid Esterase Sequence (SEQ ID NO: 99)

atggccgctccctcccgaccatgccgcccgtccggctacgaccaggtgcgcaacggcgtgccgcgcggccaggtggtgaacatctctacttctccaccgccaccaatccacccgcccggcccgcgtgtacctcccggcggtactccaaggacaagaagtactccgtgctctacctctccacggcatcggcggctccgagaacgactggtcgagggcgggcgccgcgccaacgtgatcgccgacaacctcatcgccgagggcaagatcaagccgctcatcatcgtgaccccgaacaccaacgccgcccggccgggcatcgccgacggctacgagaacttcaccaaggacctcctcaactccctcatcccgtacatcgagtccaactactccgtgtacaccgaccgcgagcaccgcgccatcgccgctctctatggcgggcgccagtccttcaacatcgccctaccaacctcgacaagttcgctacatcgccccgatctccggcccccgaacacctaccggaacgagcgccttcccggacggcggaaggccgcccgcgagaagctcaagctcctctcatcgctcgccaccaacgactccctcatcggttcggccagcgcggtgcacgagtactgcgtggccaacaacatcaaccacgtgtactggctcatccagggcgggcgccacgacttcaacgtgtggaagccggcctctggaacttctccagatggccgacgagggcgccctaccgcgcgacggcaacaccccgggtgccgaccccgtccccgaagccggccaacacccgcacgcaggccgaggactacgacggcatcaactctctccatcgagatcatcgccgtgccgcccggagggcgggcgccgcatcggtacatcacctccggcgactacctcgtgtacaagtccatcgacttcggcaacggcgccacctcctcaaggccaaggtggccaacgccaacacctccaacatcgagcttcgctcaacggcccgaacggcaccctcatcggcaccctctccgtgaagtccaccggcgactggaacacctacgaggagcagacctgctccatctccaaggtgaccggcatcaacgacctctacctcgtgttaaggcccggtgaacatcgactggttcaccttcgcggttag

Synthetic Ferulic Acid Esterase Amino Acid Sequence (SEQ ID NO: 100)

maasltmppsdydvrngvprgqvvnisyfstatnstrparvylppgyskdkkysvlyllhgiggssendwfeggggranviadnliaegkikpliivtpntnaagpgiadgyenftkdllnslipyiesnysvytdrehraiaaglsmgggqsfniqlndkfayigpisaapntypnerlfpdggkaareklklfiacgtndsligfgqrveyevanninhvywliqggghdfnvwkpglwnflqmadeagltrdgnptvptpspkpantriaedydginsssieiigvppeggrgigytsgdylvyksidfngatsfkakvanantsnielrlngpnngtligtllsvkstgdwntyeeqtsiskvtgindlylvfkpvnidwftfgv\*

13036 Sequence (SED ID NO: 101)

atggccgctccctcccgaccatgccgcccgtccggctacgaccaggtgcgcaacggcgtgccgcgcggccaggtggtgaacatctctacttctccaccgccaccaatccacccgcccggcccgcgtgtacctcccggcggtactccaaggacaagaagtactccgtgctctacctctccacggcatcggcggctccgagaacgactggtcgagggcgggcgccgcgccaacgtgatcgccgacaacctcatcgccgagggcaagatcaagccgctcatcatcgtgaccccgaacaccaacgccgcccggccgggcatcgccgacggctacgagaacttcaccaaggacctcctcaactccctcatcccgtacatcgagtccaactactccgtgtacaccgaccgcgagcaccgcgccatcgccgctctctatggcgggcgccagtccttcaacatcgccctaccaacctcgacaagttcgctacatcgccccgatctccggcccccgaacacctaccggaacgagcgccttcccggacggcggaaggccgcccgcgagaagctcaagctcctctcatcgctcgccaccaacgactccctcatcggttcggccagcgcggtgcacgagtactgcgtggccaacaacatcaaccacgtgtactggctcatccagggcgggcgccacgacttcaacgtgtggaagccggcctctggaacttctccagatggccgacgagggcgccctaccgcgcgacggcaacaccccgggtgccgaccccgtccccgaagccggccaacacccgcacgcaggccgaggactacgacggcatcaactctctccatcgagatcatcgccgtgccgcccggagggcgggcgccgcatcggtacatcacctccggcgactacctcgtgtacaagtccatcgacttcggcaacggcgccacctcctcaaggccaaggtggccaacgccaacacctccaacatcgagcttcgctcaacggcccgaacggcaccctcatcggcaccctctccgtgaagtccaccggcgactggaacacctacgaggagcagacctgctccatctccaaggtgaccggcatcaacgacctctacctcgtgttaaggcccggtgaacatcgactggttcaccttcgcggttag

13036 AA Sequence (SED ID NO: 102)

maasltmppsdydvrngvprgqvvnisyfstatnstrparvylppgyskdkkysvlyllhgiggssendwfeggggranviadnliaegkikpliivtpntnaagpgiadgyenftkdllnslipyiesnysvytdrehraiaaglsmgggqsfniqlndkfayigpisaapntypnerlfpdggkaareklklfiacgtndsligfgqrveyevanninhvywliqggghdfnvwkpglwnflqmadeagltrdgnptvptpspkpantriaedydginsssieiigvppeggrgigytsgdylvyksidfngatsfkakvanantsnielrlngpnngtligtllsvkstgdwntyeeqtsiskvtgindlylvfkpvnidwftfgv\*

13038 Sequence (SEQ ID NO: 103)

atgaggggtgtgctcgttgccctcgctctctgctcgtcgagcgccacctccatggccgcctccctcccgaccatgccgcccgtccggctacgaccaggtgcgcaacggcgtgccgcgcggccaggtggtgaacatctctacttctccaccgccaccaactccacccgcccggcccgcgtgtacctcccggcggtactccaaggacaag

aagtactcgtgctctacctcctccacggcatcggcggctccgagaacgactggttcgagggcggcggccgccaacgtgatcggcacaacctcatcggcgaggg  
caagatcaagccgctcatcatcgtgaccccggaacaccaacgccggcgccggggcatcggcgacggctacgagaacttcaccaaggacctcctcaactccctcatccc  
gtacatcgagtcgaactactcgtgtacaccgaccgagcaccgcgccatcggcgccctctctatggcgggcgccagtccttcaacatcggcctcaccaacctcgac  
aagttcgctacatcggcccgatctccgccccgaacacctaccggaacgagcgctcttcccgacggcggaaggccggccgagagaagctcaagctcctctt  
catcgctcggcaccacgactccctcatcggcttcggccagcgcggtgcacgagtactcgtggccaacaacatcaaccacgtgtactggctcatccaggcgcgcg  
ccacgacttcaacgtgtggaagccggcctctggaacttctccagatggccgacgaggccggcctcaccgcgacggcaacacccccggtgccgaccccgctcccg  
aagccggccaacacccgcatcgaggccgaggactacgacggcatcaactcctcctcatcgagatcatcggcgtgccggcgaggcgccggcgccgcatcggtac  
atcacctccggcgactacctcgtgtacaagtccatcgacttcggcaacggcgccacctcctcaaggccaaggtggccaacggcaacacctccaacatcgagcttcgcc  
tcaacggccgaacggcacctcatcggcacctctccgtgaagtccaccggcgactggaacacctacgaggagcagacctgctccatctccaaggtgaccggcatc  
aacgacctctacctcgtgttcaaggggccgggtgaacatcgactggttcaccttcggcggttag

#### 13038 AA Sequence (SEQ ID NO:104)

mrvllvalallalaasatsmaaslpmpsgydvrvngvprgqvvnisyfstatnstrparvylppgyskdkkysvlyllhgiggsendwfeggggranviadnliae  
gkikplliivtpntnaagpgiadgyenftkdlinslipyiesnysvytdrehraiaglsmgggqsfniqlnldkfayigpisaapntypnerlfpdggkaareklklfia  
cgndsligfgqrvheycvanninhvywliqggghdfnvwkpglwnflqmadeagltrdntpvptpspkpantriaedydginsssieiigvppeggrgigy  
tsdylvyksidfgngatsfkakvanantsnielrlnpgngtligtislvkstgdwntyeeqtsiskvtgindlylvfkgpvnidwftfgv\*

#### 13039 Sequence (SEQ ID NO: 105)

atgctggcgctctggccacgtcgcagctcgtcgcaacgcgcgcggcctggcggtcccgacgcgtccacgttccggcgcgccgcgccgagggcctgagggg  
ggcccgggcgctggcgcgccggcgacacgctcagcatcgcgaccagcgcgcgcgccggcgccagggcaccagcaccagcaggcgcgccgcggggcccaggttcc  
cgtcgctcgtcgtgtcgccagcgccggcgccatggcgccctccctcccgaccatcgccggctccggctacgaccaggtcgccaacggcggtcccgcgcgccaggt  
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acggcatcgcgggctccgagaacgactggttcgagggcgcgccggcgccaacgtgatcgccgacaacctcatcgccgagggcaagatcaagccgctcatcatcgt  
gaccccgaaacaccaacgccggcgccggcgccgcatcgccgacggctacgagaacttcaccaaggacctcctcaactccctcatcccgatcatcgagtcgaactactccgt  
gtacaccgaccgcgagcaccgcgccatcgccggcctctctatggcgcgcgccagtccttcaacatcggcctcaccaacctcgacaagttcgctacatcgcccgat  
ctccggcgccccgaacacctaccggaacgagcgccctctcccgacggcggaaggccggcgagagaagctcaagctcctctcatcgctcggcaccacgact  
ccctcatcggttcggccagcgcggtgcacgagtactcgtggccaacaacatcaaccacgtgtactggtcatccaggcgcgccggccacgacttcaactgtggaagc  
cgggcctctggaacttctccagatggcgacgagggcgccctcaccgcgacggcaacacccccggtgccgaccccgctcccggaagccggccaacacccgcatcg  
aggccgaggactacgacggcatcaactcctcctccatcgagatcatcgcggtgccggcgagggcgccggcgccgcatcggtacatcacctccggcgactacctcgtg  
tacaagtccatcgacttcggcaacggcgccacctcctcaaggccaaggtggccaacggcaacacctccaacatcgagcttcgctcaacggcccggaacggcacctc  
atcgccacctctccgtgaagtccaccggcgactggaacacctacgaggagcagacctgctccatctccaaggtgaccggcatcaacgacctctacctcgtgttcaagg  
gccccggtgaacatcgactggttcaccttcggcggttag

#### 13039 AA Sequence (SEQ ID NO: 106)

mlaalatsqlvatraglvpdastfrrgaagglrgarasaaadtlsmrtsaraaprhqhqqarrgarfplvvcasagamaaslpmpsgydvrvngvprgqvvn  
syfstatnstrparvylppgyskdkkysvlyllhgiggsendwfeggggranviadnliaegkikplliivtpntnaagpgiadgyenftkdlinslipyiesnysvytdre  
hraiaaglsmgggqsfniqlnldkfayigpisaapntypnerlfpdggkaareklklfiacgndsligfgqrvheycvanninhvywliqggghdfnvwkpglw  
nflqmadeagltrdntpvptpspkpantriaedydginsssieiigvppeggrgigytsdylvyksidfgngatsfkakvanantsnielrlnpgngtligtislvk  
stgdwntyeeqtsiskvtgindlylvfkgpvnidwftfgv\*

#### 13347 Sequence (SEQ ID NO: 107)

atgaggggtgtgctcgttgcctcgtctcctggtctcgtcgcgagcgccacctccatggccgcctccctcccgaccatgccggcgtccggctacgaccaggtcgcca  
acggcggtgccgcgcccaggtggtgaacatctcctacttctccaccgccaccaactccaccggccggcgccgctgtacctcccgccgggctactccaaggacaag  
aagtactcgtgctctacctcctccacggcatcggcggtccgagaacgactggttcgagggcgcgccggcgccaacgtgatcgccgacaacctcatcgccgaggg  
caagatcaagccgctcatcatcgtgaccccgaaacaccaacgccggcgccggggcatcgccgacggctacgagaacttcaccaaggacctcctcaactccctcatccc  
gtacatcgagtcgaactactcgtgtacaccgaccgagcaccgcgccatcgccggcctctctatggcgcgcgccagtccttcaacatcgccctcaccaacctcgac  
aagttcgctacatcggcccgatctccgccccgaacacctaccggaacgagcgccctcttcccgacggcggaaggccggcgagagaagctcaagctcctctt  
catcgctcggcaccacgactccctcatcggcttcggccagcgcggtgcacgagtactcgtggccaacaacatcaaccacgtgtactggtcatccaggcgcgcg  
ccacgacttcaacgtgtggaagccggcctctggaacttctccagatggccgacgagggcgccctcaccgcgacggcaacacccccggtgccgaccccgctcccg  
aagccggccaacacccgcatcgaggccgaggactacgacggcatcaactcctcctccatcgagatcatcgcggtgccggcgaggcgccggcgccgcatcggtac  
atcacctccggcgactacctcgtgtacaagtccatcgacttcggcaacggcgccacctcctcaaggccaaggtggccaacggcaacacctccaacatcgagcttcgcc

tcaacggcccgaaacggcaccctcatcggcaccctctccgtgaagtcaccggcgactggaacacctacgaggagcagacctgctccatctccaaggtgaccggcatc  
aacgacctctacctcgtgttcaagggcccggtgaacatcgactggttcaccttcggcgtgtccgagaaggacgaactctag

13347 Sequence (SEQ ID NO: 108)

mrvllvalallalaasatsmaaslptmppsdydqvrngvprgqvvnisyfstatnstrparvylppgyskdkkysvlyllhgiggssendwfeggggranviadnliae  
gkikpliivtpntnaagpgiadgyenftkdllnslipyiesnysvytdrehraiaglsmgggqsfngltnldkfayigpisaapntypnerlfpdggkaareklklfia  
cgtndsligfgqrvheycvanninhvywliqggghdfnvwkpglwnflqmadeagltrdgnptvptpspkpantrieaedydginsssieiigvppeggrgigyi  
tsgdylvyksidfgngatsfkakvanantsnielrlnpngtligtlsvkstgdwntyeeqtsiskvtgindlylvfkpvnidwftfgvsekdel\*

### Example 53

#### Hydrolytic degradation of corn fiber by ferulic acid esterase

Corn fiber is a major by-product of corn wet and dry milling. The fiber component is composed primarily of coarse fiber arising from the seed pericarp (hull) and aleurone, with a smaller fraction of fine fiber coming from the endosperm cell walls. Ferulic acid, a hydroxycinnamic acid, is found in high concentrations in the cell walls of cereal grains resulting in a cross linking of lignin, hemicellulose and cellulose components of the cell wall. Enzymatic degradation of ferulate cross-linking is an important step in the hydrolysis of corn fiber and may result in the accessibility of further enzymatic degradation by other hydrolytic enzymes.

#### Ferulic Acid Esterase Activity Assay

Ferulic acid esterase, FAE-1, (maize optimised synthetic gene from *C. thermocellum*) was expressed in *E. coli*. Cells were harvested and stored at  $-80^{\circ}\text{C}$  overnight. Harvested bacteria was suspended in 50mM Tris buffer pH7.5. Lysozyme was added to a final concentration of 200 ug/mL and the sample incubated 10 minutes at room temperature with gently shaking. The sample was centrifuged at  $4^{\circ}\text{C}$  for 15 minutes at 4000 rpm. Following centrifugation, the supernatant was transferred to a 50 mL conical tube, and placed in 70 degree Celsius water bath for 30 minutes. The sample was then centrifuged for 15 minutes at 4000 rpm and the cleared supernatant transferred to a conical tube (Blum et al. J Bacteriology, Mar 2000, pg 1346-1351.)

The recombinant FAE-1 was tested for activity using 4-methylumbelliferyl ferulate as described in Mastihubova et al (2002) Analytical Biochemistry 309 96-101. Recombinant protein FAE-1 (104-3) was diluted 10, 100, and 1000 fold and assayed. Activity assay results are shown in Figure 22.

#### Preparation of Corn Seed Fiber

Corn pericarp coarse fiber was isolated by steeping yellow dent #2 kernels for 48hrs at 50 °C in 2000 ppm sodium metabisulfite( (Aldrich). Kernels were mixed with water in equal parts and blended in a Waring laboratory heavy duty blender with the blade in reverse orientation. Blender was controlled with a variable autotransformer (Staco Energy) at 50% voltage output for 2 min. Blended material was washed with tap water over a standard test sieve #7(Fisher scientific) to separate coarse fiber from starch fractions. Coarse fiber and embryos were separated by floating the fiber way from the embryos with hot tap water in a 4L beaker (Fisher scientific). The fiber was then soaked in ethanol prior to drying overnight in a vacuum oven(Precision) at 60° C. Corn coarse fiber derived from corn kernel pericarp was milled with a laboratory mill 3100 fitted with a mill feeder 3170(Perten instruments) to 0.5mm particle size.

#### Corn Fiber Hydrolysis Assay

Course fiber (CF) was suspended in 50 mM citrate-phosphate buffer, pH 5.2 at 30 mg/ 5 ml buffer. The CF stock was vortexed and transferred to a 40 ml modular reservoir (Beckman, Cat. No. 372790). The solution was mixed well then 100 ul transferred to a 96 well plate (Corning Inc., Cat. No.9017, polystyrene, flat bottom). Enzyme was added at 1-10 ul/well and the final volume adjusted to 110 ul with buffer. CF background controls contained 10 ul of buffer only. Plates were sealed with aluminum foil and incubated at 37°C with constant shaking for 18 hours. The plates were centrifuged for 15 min at 4000 rpm. 1-10 ul of CF supernatant was transferred to a 96 well plate preloaded with 100 ul of BCA reagents (BCA-reagents: Reagent A (Pierce, Prod.# 23223), Reagent B (Pierce, Prod.# 23224). The final volume was adjusted to 110 ul. The plate was sealed with aluminum foil and placed at 85°C for 30 min. Following incubation at 85°C, the plate was centrifuged for 5 min at 2500 rpm. Absorbance



values were read at 562 nm (Molecular Devices, Spectramax Plus). Samples were quantified with D-glucose and D-xylose (Sigma) calibration curves. Assay results are reported as total sugar released.

#### Measurement of total sugar released by Ferulic Acid Esterase in Corn Seed Fiber Hydrolysis Assay

Results from the recombinant FAE-1 fiber hydrolysis assay showed no increase in total reducing sugars (data not shown). These results were not unexpected since it has been reported in the literature that an increase in total reducing sugars is detectable only when other hydrolytic enzymes are used in combination with the FAE ( Yu et al J. Agric. Food Chem. 2003, 51, 218-223). Figure 23 shows that addition of FAE-2 to a fungal supernatant which had been grown on corn fiber, shows an increase in total reducing sugars. This suggests that FAE does play an important role in corn fiber hydrolysis.

Figure 23 shows Corn Fiber Hydrolysis assay results showing increase in release of total reducing sugars from corn fiber with addition of FAE-2 to fungal supernatant (FS9).

#### Analysis of Ferulic Acid released from corn seed fiber by FAE-1

FAE activity on corn fiber was tested by following the release of ferulic acid as described in Walfron and Parr (1996) ( Waldron, KW, Parr AJ 1996 Vol 7 pages 305-312 Phytochem Anal) with slight modification. Corn coarse fiber derived from corn kernel pericarp was milled with a laboratory mill 3100 fitted with a mill feeder 3170 (Perten instruments) to 0.5mm particle size and used as substrate at a concentration of 10 mg/ml. 1 ml assays were conducted in 24 well Becton Dickenson Multiwell™. Substrate was incubated in 50 mM citrate phosphate pH 5.4 at 50° C at 110 rpm for 18 hrs in the presence and absence of recombinant FAE. After the incubation period, samples were centrifuged for 10 minutes at 13,000 rpm prior to ethyl acetate extraction. All solvents and acids used were from Fisher Scientific. 0.8 ml of supernatant was acidified with 0.5 ml acetic glacial acid and extracted three times with equivalent volume of



ethyl acetate. Organic fractions were combined and speed vac to dryness (Savant) at 40° C. Samples were then suspended with 100µl of methanol and used for HPLC analysis.

HPLC chromatography was carried out as follows. Ferulic acid (ICN Biomedicals) was used as standard in HPLC analysis (data not shown). HPLC analysis was conducted with a Hewlett Packard series 1100 HPLC system. The procedure employed a C<sub>18</sub> fully capped reverse phase column (XterraRp<sub>18</sub>, 150mm X 3.9mm i.d. 5µm particle size) operated in 1.0 ml min<sup>-1</sup> at 40°C. Ferulic Acid was eluted with a gradient of 25 to 70 % B in 32 min (solvent A: H<sub>2</sub>O, 0.01%b TFA; solvent B: MeCN, 0.0075%).

As shown in Figure 24, FA released from corn fiber was 2-3 fold higher than control when treated with 10 or 100 ul of FAE-1. These results clearly show that FAE-1 is capable of hydrolyzing corn fiber.

### Example 54

#### Functionality in fermentation of maize expressed glucoamylase and amylase

This example demonstrates that maize-expressed enzymes will support fermentation of starch in a corn slurry in the absence of added enzyme and without cooking the corn slurry. Maize kernels that contain *Rhizopus oryzae* glucoamylase (ROGA) (SEQ ID NO: 49) were produced as described in Example 32. Maize kernels that contain the barley low-pI α-amylase (AMYI) (SEQ ID NO: 88) are produced as described in Example 46. The following materials are used in this example:

*Aspergillus niger* glucoamylase (ANGA) was purchased from Sigma.

*Rhizopus* species glucoamylase (RxGA) was purchased from Wako as a dry crystalline powder and made up in 10 mM NaAcetate pH 5.2, 5 mM CaCl<sub>2</sub>. at 10 mg/ml.

MAMYI Microbially produced AMYI was prepared at approximately 0.25 mg/ml in 10 mM NaAcetate pH 5.2, 5 mM CaCl<sub>2</sub>.

Yeast was *Saccharomyces cereviceae*

YE was a sterile 5% solution of yeast extract in water

Yeast starter contained 50 g maltodextrin, 1.5 g yeast extract, 0.2 mg ZnSO<sub>4</sub> in a total volume of 300 ml of water. the medium was sterilized by autoclaving after preparation. After cooling to room temperature, 1 ml of tetracycline (10 mg/ml in ethanol), 100 µl AMG300 glucoamylase and 155 mg active dry yeast. were added. The mixture was then shaken at 30 °C for 22 h. The overnight yeast culture was diluted 1/10 with water and A600 measured to determine the yeast number, as described in Current Protocols in Molecular Biology.

ROGA flour Kernels were pooled from several T0 lines shown to have active glucoamylase The seeds were ground in the Kleco, and all flour was pooled .

AMYI flour Kernels from T0 corn expressing AMYI were pooled and ground as above.

Control flour Kernels from with similar genetic background were ground in the same fashion as the ROGA expressing corn

An inoculation mixture was prepared in a sterile tube; it contained per 1.65 ml: yeast cells (1x 10<sup>7</sup>), yeast extract (8.6 mg), tetracycline (55 µg). 1.65 ml was added / g flour to each fermentation tube.

Fermentation preparation: Flour was weighed out at 1.8 g / tube into tared 17 x 100 mm sterile polypropylene. 50 µl of 0.9 M H<sub>2</sub>SO<sub>4</sub> was added to bring the final pH prior to fermentation to 5. The inoculation mixture (2.1 ml) was added / tube. along with RXGA, AMYI-P and amylase desalting buffer as indicated below. The quantity of buffer was adjusted based on moisture content of each flour so that the total solids content was constant in each tube. The tubes were mixed thoroughly, weighed and placed into a plastic bag and incubated at 30 °C.

Table 21

Tube	Flours			Innoculation	Microbial enzymes		Amylase desalting Buffer
	Control	ROGA	AMYI		RXGA	AMYI-P	
	g	g	g	ml	ml	ml	ml
A	1.8			2.1	0	0	

B	1.8			2.1	0.036	0	1
C	1.8			2.1	0.036	1	0
D	1.8			2.1	0	1	0.036
E	1.6		0.2	2.1	0.036	0	1
F	0.2	1.6		2.1			1
G	0.2	1.6		2.1	0	1	0
H	0	1.6	0.2	2.1		0	1

The fermentation tubes were weighed at intervals over the 67 h time course. Loss of weight corresponds to evolution of CO<sub>2</sub> during fermentation. The ethanol content of the samples was determined after 67 h of fermentation by the DCL ethanol assay method. The kit (catalogue # 229-29) was purchased from Diagnostic Chemicals Limited, Charlottetown, PE, Canada, D1E 1B0. Samples (10 µl) were drawn in triplicate from each fermentation tube and diluted into 990 µl of water. 10 µl of the diluted samples were mixed with 1.25 ml of a 12.5/1 mixture of assay buffer / ADH-NAD reagent. Standards (0, 5, 10, 15 & 20% v/v ETOH) were diluted and assayed in parallel. Reactions were incubated at 37 °C for 10 min, then A340 read. Standards were prepared in duplicate, samples from each fermentation were prepared in triplicate (including the initial dilution). The weight of the samples changed with time as detailed in table below. The weight loss is expressed as a percentage of the initial sample weight at time 0.

Table 22

		Time (h)					
		0	18	24	42	48	67
Sample	Flour Composition	% wgt loss					
A	Control	0.00	8.09	9.38	12.96	13.83	16.85
B	Control + RXGA	0.00	11.48	14.20	21.79	23.83	24.63
C	Control + RXGA + MAMYI	0.00	17.90	23.27	36.48	39.07	47.59
D	Control + MAMYI	0.00	13.70	17.72	28.27	30.80	38.27
E	Control +RXGA + AMYI flour	0.00	16.85	21.60	33.95	36.98	45.74
F	ROGA flour	0.00	9.81	11.74	16.96	18.39	23.17
G	ROGA flour + MAMYI	0.00	15.53	19.69	29.75	32.11	39.94
H	ROGA flour + AMYI flour	0.00	13.35	16.27	23.60	25.53	31.68

These data show that the ROGA enzyme expressed in maize increases fermentation rate as compared to the no-enzyme control. It also confirms previous data indicating that the AMYI

enzyme expressed in maize kernels is a potent activator of fermentation of the starch in corn. The ethanol contents are detailed below.

Table 23

Sample	Flour Composition	ETOH % v/v	Standard deviation
A	Control	2.09	0.08
B	Control + RXGA	7.97	0.18
C	Control + RXGA + MAMYI	13.47	0.27
D	Control + MAMYI	11.26	0.12
E	Control +RXGA + AMYI flour	12.28	0.08
F	ROGA flour	3.55	0.05
G	ROGA flour + MAMYI	11.29	0.18
H	ROGA flour + AMYI flour	8.58	0.13

These data also demonstrate that expressing *Rhizopus oryzae* glucoamylase in maize facilitates increased fermentation of the starch in corn. Similarly, expression of the barley amylase in maize makes corn starch more fermentable with out adding exogenous enzymes.

#### **Example 55** **Cellobiohydrolase I**

The *Trichoderma reesei* cellobiohydrolase I (CBH I) gene was amplified and cloned by RT-PCR based on a published database sequence (accession # E00389). The cDNA sequence was analyzed for the presence of a signal sequence using the SignalP program, which predicted a 17 amino acid signal sequence. The DNA sequence encoding the signal sequence was replaced with an ATG by PCR, as shown in the sequence (SEQ ID NO: 79). This cDNA sequence was used to make subsequent constructs. Additional constructs are made by substituting a maize optimised version of the gene (SEQ ID NO: 93).

#### **Example 56** **Cellobiohydrolase II**

The *Trichoderma reesei* cellobiohydrolase II (CBH II) gene was amplified and cloned by RT-PCR based on a published database sequence (accession # M55080). The cDNA sequence was analyzed for the presence of a signal sequence using the SignalP program, which predicted

an 18 amino acid signal sequence. The DNA sequence encoding the signal sequence was replaced with an ATG by PCR, as shown in the sequence (SEQ ID NO: 81). This cDNA sequence was used to make subsequent constructs. Additional constructs are made by substituting a maize optimised version (SEQ ID NO: 94) of the gene.

### Example 57

#### Construction of transformation vectors for the *Trichoderma reesii* cellobiohydrolase I and cellobiohydrolase II

Cloning of the *Trichoderma reesii* cellobiohydrolase I (*cbhi*)cDNA without the native N-terminal signal sequence is described in Example 55. Expression cassettes were constructed to express the *Trichoderma reesii* cellobiohydrolase I cDNA in maize endosperm with various targeting signals as follows:

Plasmid 12392 comprises the *Trichoderma reesii cbhi* cDNA cloned behind the  $\gamma$  zein promoter for expression specifically in the endosperm for expression in the cytoplasm.

Plasmid 12391 comprises the maize  $\gamma$ -zein N-terminal signal sequence (MRVLLVALALLALAASATS)(SEQ ID NO:17) fused to *Trichoderma reesii cbhi* cDNA as described above in Example 1 for targeting to the endoplasmic reticulum and secretion into the apoplast (Torrent et al. 1997). The fusion was cloned behind the  $\gamma$  zein promoter for expression specifically in the endosperm.

Plasmid 12392 comprises the  $\gamma$ -zein N-terminal signal sequence fused to the *Trichoderma reesii cbhi* cDNA with a C-terminal addition of the sequence KDEL for targeting to and retention in the endoplasmic reticulum (ER) (Munro and Pelham, 1987). The fusion was cloned behind the maize  $\gamma$  zein promoter for expression specifically in the endosperm.

Plasmid 12656 comprises the waxy amyloplast targeting peptide (Klosgen et al., 1986) fused to the *Trichoderma reesii cbhi* cDNA for targeting to the amyloplast. The fusion was cloned behind the maize  $\gamma$  zein promoter for expression specifically in the endosperm.

All expression cassettes were moved into a binary vector (pNOV2117) for transformation into maize via *Agrobacterium* infection. The binary vector contained the phosphomannose



isomerase (PMI) gene which allows for selection of transgenic cells with mannose. Transformed maize plants were either self-pollinated or outcrossed and seed was collected for analysis.

Additional constructs (plasmids 12652, 12653, 12654 and 12655) were made with the targeting signals described above fused to *Trichoderma reesii* cellobiohydrolaseII (*cbhii*) cDNA in precisely the same manner as described for the *Trichoderma reesii* *cbhi* cDNA. These fusions were cloned behind the maize Q protein promoter (50Kd  $\gamma$  zein) (SEQ ID NO: 98) for expression specifically in the endosperm and transformed into maize as described above. Transformed maize plants were either self-pollinated or outcrossed and seed was collected for analysis.

Combinations of the enzymes can be produced either by crossing plants expressing the individual enzymes or by cloning several expression cassettes into the same binary vector to enable co-transformation.

### **Example 58**

#### **Expression of a Cbhi in corn**

T1 seed from self-pollinated maize plants transformed with either plasmid 12390, 12391 or 12392 was obtained. The 12390 construct targets the expression of the CbhI in the endoplasmic reticulum of the endosperm, the 12391 construct targets the expression of the CbhI in the apoplast of the endosperm and the 12392 construct targets the expression of the CbhI in the cytoplasm of the endosperm.

Extraction and detection of the CbhI from corn-flour: Polyclonal antibodies to CbhI and CbhII were produced in goat according to established protocols. Flour from the CbhI transgenic seeds was obtained by grinding them in an Autogizer grinder. Approximately 50 mg of flour was resuspended in 0.5ml of 20mM NaPO<sub>4</sub> buffer (pH 7.4), 150mM NaCl followed by incubation for 15 minutes at RT with continuous shaking. The incubated mixture was then spun for 10min. at 10,000xg. The supernatant was used as enzyme source. 30  $\mu$ l of this extract was loaded on a 4-12 % NuPAGE gel (invitrogen) and separated in the NuPAGE MES running buffer (invitrogen). Protein was blotted onto nitrocellulose membranes and Western blot

analysis was done following established protocols using the specific antibodies described above followed by alkaline phosphatase conjugated rabbit antigoat IgG (H+L) . Alkaline phosphatase activity was detected by incubation of the membranes with ready to use BCIP/MBT (plus) substrate from Moss Inc.

Western Blot analysis was done of T1 seeds from different events transformed with plasmid 12390. Expression of CbhI protein was compared to the non-transgenic control, and was detected in a number of events.

The Cracked Corn Assay was performed essentially as described in Example 49, using transgenic seed expressing Cbhi. Starch recovery from the transgenic seed was measured and the results are set forth in Table 24.

Table 24.

Conditions	Line 3-non expressing control	Line 4- CBHI expressing
	Starch (mg)	
400ppm SO2-No Bromelain	40.2	78.1
400ppmSO2-Plus Bromelain	48.1	118.7
2000ppm SO2-No Bromelain	47.5	73.1
2000ppmSO2-Plus Bromelain	49.2	109

### Example 59

#### Preparation of Endoglucanase I Constructs

A *Trichoderma reesei* endoglucanase I (EGLI) gene was amplified and cloned by PCR based on a published database sequence (Accession # M15665; Penttila et al., 1986). Because only genomic sequences could be obtained, the cDNA was generated from the genomic sequence by removing 2 introns using Overlap PCR. The resulting cDNA sequence was analyzed for the presence of a signal sequence using the SignalP program, which predicted a 22 amino acid signal sequence. The DNA sequence encoding the signal sequence was replaced with an ATG by PCR, as shown in the sequence (SEQ ID NO: 83). This cDNA sequence was used to make subsequent constructs as set forth below.

### Overlap PCR

Overlap PCR is a technique (Ho et al., 1989) used to fuse complementary ends of two or more PCR products, and can be used to make base pair (bp) changes, add bp, or delete bp. At the site of the intended bp change, forward and reverse mutagenic primers (Mut-F and Mut-R) are made that contain the intended change and 15 bp of sequence on either side of the change. For example, to remove an intron, the primers would consist of the final 15 bp of exon 1 fused to the first 15 bp of exon 2. Primers are also prepared that anneal to the ends of the sequence to be amplified, e.g ATG and STOP codon primers. PCR amplification of the products proceeds with the ATG/Mut-R primer pair and the Mut-F/STOP primer pair in independent reactions. The products are gel purified and fused together in a PCR without added primers. The fusion reaction is separated on a gel, and the band of the correct size is gel purified and cloned. Multiple changes can be accomplished simultaneously through the addition of additional mutagenic primer pairs.

### EGLI Plant Expression Constructs

Expression cassettes were made to express the *Trichoderma reesei* EGLI cDNA in maize endosperm as follows:

**13025** comprises the *T. reesei* EGLI gene cloned behind the maize  $\gamma$ -zein promoter for cytoplasmic localization and expression specifically in the endosperm.

**13026** comprises the maize  $\gamma$ -zein N-terminal signal peptide (MRVLLVALALLALAASATS) fused to the *T. reesei* EGLI gene for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**13027** comprises the maize  $\gamma$ -zein N-terminal signal peptide fused to the *T. reesei* EGLI gene with a C-terminal addition of the sequence KDEL for targeting to and retention in the endoplasmic reticulum. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**13028** comprises the maize Granule Bound Starch Synthase I (GBSSI) N-terminal signal peptide (N-terminal 77 amino acids) fused to the *T. reesei* EGLI gene for targeting to the lumen of the amyloplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**13029** comprises the maize GBSSI N-terminal signal peptide fused to the *T. reesei* EGLI gene with a C-terminal addition of the starch binding domain (C-terminal 301 amino acids) of the maize GBSSI gene for targeting to the starch granule. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

Additional Expression cassettes are generated using a maize optimised version of EGLI (SEQ ID NO: 95)

#### **EGLI Enzyme Assays**

EGLI enzyme activity is measured in maize transgenics using the Malt Beta-Glucanase Assay Kit (Cat # K-MBGL) (Megazyme International Ireland Ltd.) The enzymatic activity of EGL I expressors is tested in the Corn Fiber Hydrolysis Assay as described in Example 53.

**Example 60**  
 **$\beta$ -Glucosidase 2**

A *Trichoderma reesei*  $\beta$ -Glucosidase 2 (BGL2) gene was amplified and cloned by RT-PCR based on sequence Accession # AB003110 (Takashima et al., 1999).

**BGL2 Plant Expression Constructs**

Expression cassettes were made to express the *Trichoderma reesei* BGL2 cDNA (SEQ ID NO: 89) in maize endosperm as follows:

**13030** comprises the *T. reesei* BGL2 gene cloned behind the maize  $\gamma$ -zein promoter for cytoplasmic localization and expression specifically in the endosperm.

**13031** comprises the maize  $\gamma$ -zein N-terminal signal peptide (MRVLLVALALLALAASATS) fused to the *T. reesei* BGL2 gene for targeting to the endoplasmic reticulum and secretion into the apoplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**13032** comprises the maize  $\gamma$ -zein N-terminal signal peptide fused to the *T. reesei* BGL2 gene with a C-terminal addition of the sequence KDEL for targeting to and retention in the endoplasmic reticulum. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**13033** comprises the maize Granule Bound Starch Synthase I (GBSSI) N-terminal signal peptide (N-terminal 77 amino acids) fused to the *T. reesei* BGL2 gene for targeting to the lumen of the amyloplast. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

**13034** comprises the maize GBSSI N-terminal signal peptide fused to the *T. reesei* BGL2 gene with a C-terminal addition of the starch binding domain (C-terminal 301 amino acids) of the



maize GBSSI gene for targeting to the starch granule. The fusion was cloned behind the maize  $\gamma$ -zein promoter for expression specifically in the endosperm.

Additional Expression cassettes are generated by substituting a maize optimized version of BGL2 (SEQ ID NO: 96).

All expression cassettes are inserted into the binary vector pNOV2117 for transformation into maize via *Agrobacterium* infection. The binary vector contained the phosphomannose isomerase (PMI) gene which allows for selection of transgenic cells with mannose. Transformed maize plants were either self-pollinated or outcrossed and seed was collected for analysis.

#### BGL2 Enzyme Assays

BGL2 enzyme activity is measured in transgenic maize using a protocol modified from Bauer and Kelly (Bauer, M.W. and Kelly, R.M. 1998. The family 1  $\beta$ -glucosidases from *Pyrococcus furiosus* and *Agrobacterium faecalis* share a common catalytic mechanism. Biochemistry 37: 17170-17178). The protocol can be modified to incubate samples at 37°C instead of 100°C. The enzymatic activity of BGL2-expressors is tested in the Fiber Hydrolysis Assay.

**Example 61** **$\beta$ -Glucosidase D**

The *Trichoderma reesei*  $\beta$ -Glucosidase D (CEL3D) gene was amplified and cloned by PCR based on a published database sequence (accession # AY281378; Foreman et al., 2003). Because only genomic sequences could be obtained, the cDNA was generated from the genomic sequence by removing an intron using Overlap PCR, as described in Example 58. The resulting cDNA (SEQ ID NO: 91) may be used for subsequent constructs. A maize optimised version (SEQ ID NO: 97) of the resulting cDNA may also be used for constructs.

Plant constructs can be generated and  $\beta$ -glucosidase assays can be performed as described for BGL2 in Example 60, replacing BGL2 with CEL3D.

**Example 62****Lipases**

cDNAs encoding lipases are generated using sequences from Accession # D85895, AF04488, and AF04489 (Tsuchiya et al., 1996; Yu et al., 2003) and methodology set forth in Examples 59-60.

Lipase enzyme activity can be measured in transgenic maize using the Fluorescent Lipase Assay Kit (Cat # M0612)(Marker Gene Technologies, Inc.). Lipase activity can also be measured *in vivo* using the fluorescent substrate 1,2-dioleoyl-3-(pyren-1-yl)decanoyl-*rac* glycerol (M0258), also from Marker Gene Technologies, Inc.

**Example 63****Expression of Phytase in Rice**

Vectors 11267 and 11268 comprise binary vectors that encode Nov9x phytase. Expression of the Nov9x phytase gene in both vectors is under the control of the rice glutelin-1 promoter (SEQ ID NO:67). Vectors 11267 and 11268 are derived from pNOV2117.

The Nov9x phytase expression cassette in vector 11267 comprises the rice glutelin-1 promoter, the Nov9x phytase gene with apoplast targeting signal, a PEPC intron, and the 35S terminator. The product of the Nov9x phytase coding sequence in vector 11267 is shown in SEQ ID NO: 110 .

The Nov9x phytase expression cassette in vector 11268 comprises the rice glutelin-1 promoter, the Nov9x phytase gene with ER retention (SEQ ID NO:111), a PEPC intron, and the 35S terminator. The product of the Nov9x phytase coding sequence in vector 11268 is shown in SEQ ID NO: 112.

**11267 Nov9x phytase with apoplast targeting DNA sequence (SEQ ID NO: 109).  
Translation start and stop codons are underlined. The sequence encoding the signal  
sequence of the 27-kD gamma-zein protein is in bold.**

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**11267 Nov9x phytase with apoplast targeting gene product (SEQ ID NO:110). The signal  
sequence of the 27-kD gamma-zein protein is in bold.**

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**11268 Nov9x phytase with ER retention DNA sequence (SEQ ID NO:111). The sequence  
encoding the signal sequence of the 27-kD gamma-zein protein is in bold. The sequence  
encoding the SEKDEL hexapeptide ER retention signal is underlined.**

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**11268 Nov9x phytase with ER retention, gene product (SEQ ID NO: 112). The signal sequence of the 27-kD gamma-zein protein is in bold. The ER retention signal is underlined.**

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#### Generation of transgenic rice plants

Rice (*Oryza sativa*) is used for generating transgenic plants. Various rice cultivars can be used (Hiei et al., 1994, *Plant Journal* 6:271-282; Dong et al., 1996, *Molecular Breeding* 2:267-276; Hiei et al., 1997, *Plant Molecular Biology*, 35:205-218). Also, the various media constituents described below may be either varied in concentration or substituted. Embryogenic responses are initiated and/or cultures are established from mature embryos by culturing on MS-CIM medium (MS basal salts, 4.3 g/liter; B5 vitamins (200 x), 5 ml/liter; Sucrose, 30 g/liter; proline, 500 mg/liter; glutamine, 500 mg/liter; casein hydrolysate, 300 mg/liter; 2,4-D (1 mg/ml), 2 ml/liter; adjust pH to 5.8 with 1 N KOH; Phytigel, 3 g/liter). Either mature embryos at the initial stages of culture response or established culture lines are inoculated and co-cultivated with the *Agrobacterium* strain LBA4404 containing the desired vector construction. *Agrobacterium* is cultured from glycerol stocks on solid YPC medium (100 mg/L spectinomycin and any other appropriate antibiotic) for ~2 days at 28 °C. *Agrobacterium* is re-suspended in liquid MS-CIM medium. The *Agrobacterium* culture is diluted to an OD600 of 0.2-0.3 and acetosyringone is added to a final concentration of 200 µM. *Agrobacterium* is induced with acetosyringone before mixing the solution with the rice cultures. For inoculation, the cultures are immersed in the bacterial suspension. The liquid bacterial suspension is removed and the inoculated cultures are placed on co-cultivation medium and incubated at 22°C for two days. The cultures are then transferred to MS-CIM medium with Ticarcillin (400 mg/liter) to inhibit the growth of *Agrobacterium*. For constructs utilizing the PMI selectable marker gene (Reed et al., *In Vitro Cell. Dev. Biol.-Plant* 37:127-132), cultures are transferred to selection medium containing



Mannose as a carbohydrate source (MS with 2% Mannose, 300 mg/liter Ticarcillin) after 7 days, and cultured for 3-4 weeks in the dark. Resistant colonies are then transferred to regeneration induction medium (MS with no 2,4-D, 0.5 mg/liter IAA, 1 mg/liter zeatin, 200 mg/liter Ticarcillin 2% Mannose and 3% Sorbitol) and grown in the dark for 14 days. Proliferating colonies are then transferred to another round of regeneration induction media and moved to the light growth room. Regenerated shoots are transferred to GA7-1 medium (MS with no hormones and 2% Sorbitol) for 2 weeks and then moved to the greenhouse when they are large enough and have adequate roots. Plants are transplanted to soil in the greenhouse and grown to maturity.

#### Example 64

##### Analysis of Transgenic Rice Seed Expressing Nov9X Phytase

##### ELISA For The Quantitation Of Nov9X Phytase From Rice Seed

Quantitation of phytase expressed in transgenic rice seed was assayed by ELISA. One (1g) rice seed was ground to flour in a Kleco seed grinder. 50 mg of flour was resuspended in the sodium acetate buffer described in example – for Nov9X phytase activity assay and diluted as required for the immunoassay. The Nov9X immunoassay is a quantitative sandwich assay for the detection of phytase that employs two polyclonal antibodies. The rabbit antibody was purified using protein A, and the goat antibody was immunoaffinity purified against recombinant phytase (Nov9X) protein produced in *E.coli* inclusion bodies. Using these highly specific antibodies, the assay can measure picogram levels of phytase in transgenic plants. There are three basic parts to the assay. The phytase protein in the sample is captured onto the solid phase microtiter well using the rabbit antibody. Then a “sandwich” is formed between the solid phase antibody, the phytase protein, and the secondary antibody that has been added to the well. After a wash step, where unbound secondary antibody has been removed, the bound antibody is detected using an alkaline phosphatase-labeled antibody. Substrate for the enzyme is added and color development is measured by reading the absorbance of each well. The standard curve uses a four-parameter curve fit to plot the concentrations versus the absorbance.

##### Phytase activity assay

Determination of phytase activity, based upon the estimation of inorganic phosphate released on hydrolysis of phytic acid, can be performed at 37°C following the method of Engelen, A.J. et al., *J. AOAC, Inter.*, 84, 629 (2001). One unit of enzyme activity is defined as the amount of enzyme that liberates 1  $\mu\text{mol}$  of inorganic phosphate per minute under assay conditions. For example, phytase activity may be measured by incubating 2.0 ml of the enzyme preparation with 4.0 ml of 9.1 mM sodium phytate in 250 mM sodium acetate buffer pH 5.5, supplemented with 1 mM  $\text{CaCl}_2$  for 60 minutes at 37°C. After incubation, the reaction is stopped by adding 4.0 ml of a color-stop reagent consisting of equal parts of a 10% (w/v) ammonium molybdate and a 0.235% (w/v) ammonium vanadate stock solution. Precipitate is removed by centrifugation, and phosphate released is measured against a set of phosphate standards spectrophotometrically at 415 nm. Phytase activity is calculated by interpolating the A415 nm absorbance values obtained for phytase containing samples using the generated phosphate standard curve.

This procedure may be scaled down to accommodate smaller volumes and adapted to preferred containers. Preferred containers include glass test tubes and plastic microplates. Partial submersion of the reaction vessel(s) in a water bath is essential to maintain constant temperature during the enzyme reaction.

Table 24

Trans-genic line	$\mu\text{g}$ phytase/g flour*	Phytase activity units per g flour**	Endogenous inorganic phosphate released by cooking of dehusked rice seed ( $\mu\text{mol/gseed}$ )	Endogenous inorganic phosphate released by cooking of dehusked, polished rice seed ( $\mu\text{mol/gseed}$ )
Wild type	0	0	1.442	0.469
1	510	916	1.934	0.840
2	1518	2800	2.894	1.073

\* $\mu\text{g}$  phytase was assayed by a sandwich ELISA

\*\*Phytase activity was assayed by Phytase activity assay as described above.

#### Assay of Inorganic Phosphate Release During Cooking of Transgenic Rice Expressing Phytase

Two samples of 1g seed from selected rice transgenic lines and a control wildtype line was dehusked using a benchtop Kett TR200 automatic rice husker . One sample was then

polished for 30 seconds in a Kett Rice polisher. Two volumes of H<sub>2</sub>O was added to each sample and the rice was cooked by immersing the tubes into a beaker of water. The water was brought to a boil and held in a full rolling boil for 10 minutes. The “cooked” rice seed was then ground to a paste with water bringing the total volume of the slurry to 6 ml. The slurry was centrifuged at 15,000xg for 10 minutes and the clear supernatant assayed for released endogenous inorganic phosphate. The assay of released phosphate is based on color formation as a result of molybdate and vanadate ions complexing with inorganic phosphate and is measured spectrophotometrically at 415nm as described in example – for phytase enzymatic activity. The results are in Table 24.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

#### SEQUENCE LISTING

<110> Lanahan, Mike  
<120> Self-processing Plants and Plant Parts  
<130> 109846.317  
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&lt;210&gt; 5

&lt;211&gt; 693

&lt;212&gt; PRT

&lt;213&gt; Sulfolobus solfataricus

&lt;400&gt; 5

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Ile Gly Glu Pro Phe Pro Pro Ile Glu Phe Pro Leu Glu Gln Lys Ile
      20              25              30
Ser Ser Asn Lys Ser Leu Ser Glu Leu Gly Leu Thr Ile Val Gln Gln
      35              40              45
Gly Asn Lys Val Ile Val Glu Lys Ser Leu Asp Leu Lys Glu His Ile
 50              55              60
Ile Gly Leu Gly Glu Lys Ala Phe Glu Leu Asp Arg Lys Arg Lys Arg
65              70              75              80
Tyr Val Met Tyr Asn Val Asp Ala Gly Ala Tyr Lys Lys Tyr Gln Asp
      85              90              95

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Val	Glu	Phe	Tyr	Val	Ile	Glu	Gly	Pro	Arg	Ile	Glu	Asp	Val	Leu	Glu
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Lys	Tyr	Thr	Glu	Leu	Thr	Gly	Lys	Pro	Phe	Leu	Pro	Pro	Met	Trp	Ala
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Phe	Gly	Tyr	Met	Ile	Ser	Arg	Tyr	Ser	Tyr	Tyr	Pro	Gln	Asp	Lys	Val
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Val	Glu	Leu	Val	Asp	Ile	Met	Gln	Lys	Glu	Gly	Phe	Arg	Val	Ala	Gly
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Lys	Arg	Asn	Val	Lys	Leu	Ile	Thr	Ile	Val	Asp	His	Gly	Ile	Arg	Val
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Ile	Glu	Ser	Gly	Glu	Leu	Phe	Val	Gly	Lys	Met	Trp	Pro	Gly	Thr	Thr
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Val	Tyr	Pro	Asp	Phe	Phe	Arg	Glu	Asp	Thr	Arg	Glu	Trp	Trp	Ala	Gly
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Met	Asn	Glu	Pro	Thr	Asp	Phe	Ser	Arg	Ala	Ile	Glu	Ile	Arg	Asp	Val
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Leu	Ser	Ser	Leu	Pro	Val	Gln	Phe	Arg	Asp	Asp	Arg	Leu	Val	Thr	Thr
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Phe	Pro	Asp	Asn	Val	Val	His	Tyr	Leu	Arg	Gly	Lys	Arg	Val	Lys	His
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Glu	Lys	Val	Arg	Asn	Ala	Tyr	Pro	Leu	Tyr	Glu	Ala	Met	Ala	Thr	Phe
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Phe	Gln	Gly	Arg	Asn	Phe	Ala	Glu	Ile	Asp	Asn	Ser	Met	Asp	Leu	Leu
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Lys	Ala	Thr	Asp	Gly	Ile	Asp	Thr	Glu	Pro	Val	Phe	Leu	Pro	Asp	Tyr
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Tyr	Lys	Glu	Lys	Val	Lys	Glu	Ile	Val	Glu	Leu	Arg	Tyr	Lys	Phe	Leu
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Pro	Tyr	Ile	Tyr	Ser	Leu	Ala	Leu	Glu	Ala	Ser	Glu	Lys	Gly	His	Pro
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 Ile Val Ser Lys Glu Glu Ser Arg Leu Val Thr Leu Pro Arg Gly Lys  
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 580 585 590  
 Lys Ser Thr His Glu Leu Pro Ile Tyr Leu Arg Glu Gly Ser Ile Ile  
 595 600 605  
 Pro Leu Glu Gly Asp Glu Leu Ile Val Tyr Gly Glu Thr Ser Phe Lys  
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 Arg Tyr Asp Asn Ala Glu Ile Thr Ser Ser Ser Asn Glu Ile Lys Phe  
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 Ser Arg Glu Ile Tyr Val Ser Lys Leu Thr Ile Thr Ser Glu Lys Pro  
 645 650 655  
 Val Ser Lys Ile Ile Val Asp Asp Ser Lys Glu Ile Gln Val Glu Lys  
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 Lys Ile Asn Leu Glu  
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&lt;210&gt; 6

&lt;211&gt; 2082

&lt;212&gt; DNA

<213> *Sulfolobus solfataricus*

&lt;400&gt; 6

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&lt;210&gt; 7

&lt;211&gt; 1818

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 7

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gagaacgtgg ccgcgccc

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&lt;210&gt; 8

<211> 606  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 8  
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 Gly Val Pro Asp Ala Ser Thr Phe Arg Arg Gly Ala Ala Gln Gly Leu  
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 Arg Gly Ala Arg Ala Ser Ala Ala Ala Asp Thr Leu Ser Met Arg Thr  
 35 40 45  
 Ser Ala Arg Ala Ala Pro Arg His Gln His Gln Gln Ala Arg Arg Gly  
 50 55 60  
 Ala Arg Phe Pro Ser Leu Val Val Cys Ala Ser Ala Gly Met Asn Val  
 65 70 75 80  
 Val Phe Val Gly Ala Glu Met Ala Pro Trp Ser Lys Thr Gly Gly Leu  
 85 90 95  
 Gly Asp Val Leu Gly Gly Leu Pro Pro Ala Met Ala Ala Asn Gly His  
 100 105 110  
 Arg Val Met Val Val Ser Pro Arg Tyr Asp Gln Tyr Lys Asp Ala Trp  
 115 120 125  
 Asp Thr Ser Val Val Ser Glu Ile Lys Met Gly Asp Gly Tyr Glu Thr  
 130 135 140  
 Val Arg Phe Phe His Cys Tyr Lys Arg Gly Val Asp Arg Val Phe Val  
 145 150 155 160  
 Asp His Pro Leu Phe Leu Glu Arg Val Trp Gly Lys Thr Glu Glu Lys  
 165 170 175  
 Ile Tyr Gly Pro Val Ala Gly Thr Asp Tyr Arg Asp Asn Gln Leu Arg  
 180 185 190  
 Phe Ser Leu Leu Cys Gln Ala Ala Leu Glu Ala Pro Arg Ile Leu Ser  
 195 200 205  
 Leu Asn Asn Asn Pro Tyr Phe Ser Gly Pro Tyr Gly Glu Asp Val Val  
 210 215 220  
 Phe Val Cys Asn Asp Trp His Thr Gly Pro Leu Ser Cys Tyr Leu Lys  
 225 230 235 240  
 Ser Asn Tyr Gln Ser His Gly Ile Tyr Arg Asp Ala Lys Thr Ala Phe  
 245 250 255  
 Cys Ile His Asn Ile Ser Tyr Gln Gly Arg Phe Ala Phe Ser Asp Tyr  
 260 265 270  
 Pro Glu Leu Asn Leu Pro Glu Arg Phe Lys Ser Ser Phe Asp Phe Ile  
 275 280 285  
 Asp Gly Tyr Glu Lys Pro Val Glu Gly Arg Lys Ile Asn Trp Met Lys  
 290 295 300  
 Ala Gly Ile Leu Glu Ala Asp Arg Val Leu Thr Val Ser Pro Tyr Tyr  
 305 310 315 320  
 Ala Glu Glu Leu Ile Ser Gly Ile Ala Arg Gly Cys Glu Leu Asp Asn  
 325 330 335  
 Ile Met Arg Leu Thr Gly Ile Thr Gly Ile Val Asn Gly Met Asp Val  
 340 345 350  
 Ser Glu Trp Asp Pro Ser Arg Asp Lys Tyr Ile Ala Val Lys Tyr Asp  
 355 360 365

Val	Ser	Thr	Ala	Val	Glu	Ala	Lys	Ala	Leu	Asn	Lys	Glu	Ala	Leu	Gln
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Ile	Gly	Arg	Leu	Glu	Glu	Gln	Lys	Gly	Pro	Asp	Val	Met	Ala	Ala	Ala
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Ile	Pro	Gln	Leu	Met	Glu	Met	Val	Glu	Asp	Val	Gln	Ile	Val	Leu	Leu
			420					425					430		
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Gly	Lys	Thr	Gly	Phe	His	Met	Gly	Arg	Leu	Ser	Val	Asp	Cys	Asn	Val
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Met	Ile	Gln	Asp	Leu	Ser	Trp	Lys	Gly	Pro	Ala	Lys	Asn	Trp	Glu	Asn
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Val	Leu	Leu	Ser	Leu	Gly	Val	Ala	Gly	Gly	Glu	Pro	Gly	Val	Glu	Gly
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Glu	Glu	Ile	Ala	Pro	Leu	Ala	Lys	Glu	Asn	Val	Ala	Ala	Pro		
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&lt;210&gt; 9

&lt;211&gt; 2223

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 9

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2223

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 Gln Lys Ile Pro Glu Trp Tyr Asp Ala Gly Ile Ser Ala Ile Trp Ile  
 35 40 45  
 Pro Pro Ala Ser Lys Gly Met Ser Gly Gly Tyr Ser Met Gly Tyr Asp  
 50 55 60  
 Pro Tyr Asp Tyr Phe Asp Leu Gly Glu Tyr Tyr Gln Lys Gly Thr Val  
 65 70 75 80  
 Glu Thr Arg Phe Gly Ser Lys Gln Glu Leu Ile Asn Met Ile Asn Thr  
 85 90 95  
 Ala His Ala Tyr Gly Ile Lys Val Ile Ala Asp Ile Val Ile Asn His  
 100 105 110  
 Arg Ala Gly Gly Asp Leu Glu Trp Asn Pro Phe Val Gly Asp Tyr Thr  
 115 120 125  
 Trp Thr Asp Phe Ser Lys Val Ala Ser Gly Lys Tyr Thr Ala Asn Tyr  
 130 135 140  
 Leu Asp Phe His Pro Asn Glu Leu His Ala Gly Asp Ser Gly Thr Phe

145					150					155					160
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			180					185					190		
Ile	Asp	Ala	Trp	Arg	Phe	Asp	Tyr	Val	Lys	Gly	Tyr	Gly	Ala	Trp	Val
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Val	Lys	Asp	Trp	Leu	Asn	Trp	Trp	Gly	Gly	Trp	Ala	Val	Gly	Glu	Tyr
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Trp	Asp	Thr	Asn	Val	Asp	Ala	Leu	Leu	Asn	Trp	Ala	Tyr	Ser	Ser	Gly
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Cys	Gly	Val	Gly	Thr	Ser	Ile	Ala	Gly	Ile	Leu	Glu	Ala	Asp	Arg	Val
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Tyr	Ile	Ala	Val	Lys	Tyr	Asp	Val	Ser	Thr	Ala	Val	Glu	Ala	Lys	Ala
			500					505					510		
Leu	Asn	Lys	Glu	Ala	Leu	Gln	Ala	Glu	Val	Gly	Leu	Pro	Val	Asp	Arg
	515						520					525			
Asn	Ile	Pro	Leu	Val	Ala	Phe	Ile	Gly	Arg	Leu	Glu	Glu	Gln	Lys	Gly
	530					535					540				
Pro	Asp	Val	Met	Ala	Ala	Ala	Ile	Pro	Gln	Leu	Met	Glu	Met	Val	Glu
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Asp	Val	Gln	Ile	Val	Leu	Leu	Gly	Thr	Gly	Lys	Lys	Lys	Phe	Glu	Arg
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<211> 1515
<212> DNA
<213> Zea mays
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1515

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 <213> Zea mays

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 aattgcacgt caaggggtatt gggtaagaaa caatcaaaca aatcctctct gtgtgcaaag 180  
 aaacacggtg agtcatgccg agatcatact catctgatat acatgcttac agctcacaag 240  
 acattacaaa caactcatat tgcattacaa agatcgtttc atgaaaaata aaataggccg 300  
 gacaggacaa aaatccttga cgtgtaaagt aaatttataa caaaaaaaaa gccatatgtc 360  
 aagctaaatc taattcgttt tacgtagatc aacaacctgt agaaggcaac aaaactgagc 420  
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 gagtcatata catttggtgcaa gaaacatga agctgcctac agccgtctcg gtggcataag 540  
 aacacaagaa attgtgttaa ttaatcaaag ctataaataa cgctcgcatg cctgtgcact 600  
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 <223> synthetic

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 Gln Ala Phe Tyr Trp Asp Val Pro Ser Gly Gly Ile Trp Trp Asp Thr  
 35 40 45  
 Ile Arg Gln Lys Ile Pro Glu Trp Tyr Asp Ala Gly Ile Ser Ala Ile  
 50 55 60  
 Trp Ile Pro Pro Ala Ser Lys Gly Met Ser Gly Gly Tyr Ser Met Gly  
 65 70 75 80  
 Tyr Asp Pro Tyr Asp Tyr Phe Asp Leu Gly Glu Tyr Tyr Gln Lys Gly  
 85 90 95  
 Thr Val Glu Thr Arg Phe Gly Ser Lys Gln Glu Leu Ile Asn Met Ile  
 100 105 110  
 Asn Thr Ala His Ala Tyr Gly Ile Lys Val Ile Ala Asp Ile Val Ile  
 115 120 125  
 Asn His Arg Ala Gly Gly Asp Leu Glu Trp Asn Pro Phe Val Gly Asp  
 130 135 140  
 Tyr Thr Trp Thr Asp Phe Ser Lys Val Ala Ser Gly Lys Tyr Thr Ala  
 145 150 155 160  
 Asn Tyr Leu Asp Phe His Pro Asn Glu Leu His Ala Gly Asp Ser Gly  
 165 170 175  
 Thr Phe Gly Gly Tyr Pro Asp Ile Cys His Asp Lys Ser Trp Asp Gln  
 180 185 190

Tyr Trp Leu Trp Ala Ser Gln Glu Ser Tyr Ala Ala Tyr Leu Arg Ser  
 195 200 205  
 Ile Gly Ile Asp Ala Trp Arg Phe Asp Tyr Val Lys Gly Tyr Gly Ala  
 210 215 220  
 Trp Val Val Lys Asp Trp Leu Asn Trp Trp Gly Gly Trp Ala Val Gly  
 225 230 235 240  
 Glu Tyr Trp Asp Thr Asn Val Asp Ala Leu Leu Asn Trp Ala Tyr Ser  
 245 250 255  
 Ser Gly Ala Lys Val Phe Asp Phe Pro Leu Tyr Tyr Lys Met Asp Ala  
 260 265 270  
 Ala Phe Asp Asn Lys Asn Ile Pro Ala Leu Val Glu Ala Leu Lys Asn  
 275 280 285  
 Gly Gly Thr Val Val Ser Arg Asp Pro Phe Lys Ala Val Thr Phe Val  
 290 295 300  
 Ala Asn His Asp Thr Asp Ile Ile Trp Asn Lys Tyr Pro Ala Tyr Ala  
 305 310 315 320  
 Phe Ile Leu Thr Tyr Glu Gly Gln Pro Thr Ile Phe Tyr Arg Asp Tyr  
 325 330 335  
 Glu Glu Trp Leu Asn Lys Asp Lys Leu Lys Asn Leu Ile Trp Ile His  
 340 345 350  
 Asp Asn Leu Ala Gly Gly Ser Thr Ser Ile Val Tyr Tyr Asp Ser Asp  
 355 360 365  
 Glu Met Ile Phe Val Arg Asn Gly Tyr Gly Ser Lys Pro Gly Leu Ile  
 370 375 380  
 Thr Tyr Ile Asn Leu Gly Ser Ser Lys Val Gly Arg Trp Val Tyr Val  
 385 390 395 400  
 Pro Lys Phe Ala Gly Ala Cys Ile His Glu Tyr Thr Gly Asn Leu Gly  
 405 410 415  
 Gly Trp Val Asp Lys Tyr Val Tyr Ser Ser Gly Trp Val Tyr Leu Glu  
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 <212> PRT  
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 <223> synthetic

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 35 40 45  
 Ile Arg Gln Lys Ile Pro Glu Trp Tyr Asp Ala Gly Ile Ser Ala Ile  
 50 55 60  
 Trp Ile Pro Pro Ala Ser Lys Gly Met Ser Gly Gly Tyr Ser Met Gly

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Thr	Val	Glu	Thr	Arg	Phe	Gly	Ser	Lys	Gln	Glu	Leu	Ile	Asn	Met	Ile
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Asn	Thr	Ala	His	Ala	Tyr	Gly	Ile	Lys	Val	Ile	Ala	Asp	Ile	Val	Ile
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Asn	His	Arg	Ala	Gly	Gly	Asp	Leu	Glu	Trp	Asn	Pro	Phe	Val	Gly	Asp
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Tyr	Thr	Trp	Thr	Asp	Phe	Ser	Lys	Val	Ala	Ser	Gly	Lys	Tyr	Thr	Ala
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Asn	Tyr	Leu	Asp	Phe	His	Pro	Asn	Glu	Leu	His	Ala	Gly	Asp	Ser	Gly
			165					170						175	
Thr	Phe	Gly	Gly	Tyr	Pro	Asp	Ile	Cys	His	Asp	Lys	Ser	Trp	Asp	Gln
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Tyr	Trp	Leu	Trp	Ala	Ser	Gln	Glu	Ser	Tyr	Ala	Ala	Tyr	Leu	Arg	Ser
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Trp	Val	Val	Lys	Asp	Trp	Leu	Asn	Trp	Trp	Gly	Gly	Trp	Ala	Val	Gly
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Ala	Phe	Asp	Asn	Lys	Asn	Ile	Pro	Ala	Leu	Val	Glu	Ala	Leu	Lys	Asn
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Gly	Gly	Thr	Val	Val	Ser	Arg	Asp	Pro	Phe	Lys	Ala	Val	Thr	Phe	Val
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Ala	Asn	His	Asp	Thr	Asp	Ile	Ile	Trp	Asn	Lys	Tyr	Pro	Ala	Tyr	Ala
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Phe	Ile	Leu	Thr	Tyr	Glu	Gly	Gln	Pro	Thr	Ile	Phe	Tyr	Arg	Asp	Tyr
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Glu	Glu	Trp	Leu	Asn	Lys	Asp	Lys	Leu	Lys	Asn	Leu	Ile	Trp	Ile	His
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Asp	Asn	Leu	Ala	Gly	Gly	Ser	Thr	Ser	Ile	Val	Tyr	Tyr	Asp	Ser	Asp
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Glu	Met	Ile	Phe	Val	Arg	Asn	Gly	Tyr	Gly	Ser	Lys	Pro	Gly	Leu	Ile
	370					375					380				
Thr	Tyr	Ile	Asn	Leu	Gly	Ser	Ser	Lys	Val	Gly	Arg	Trp	Val	Tyr	Val
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Pro	Lys	Phe	Ala	Gly	Ala	Cys	Ile	His	Glu	Tyr	Thr	Gly	Asn	Leu	Gly
			405						410					415	
Gly	Trp	Val	Asp	Lys	Tyr	Val	Tyr	Ser	Ser	Gly	Trp	Val	Tyr	Leu	Glu
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Ala	Pro	Ala	Tyr	Asp	Pro	Ala	Asn	Gly	Gln	Tyr	Gly	Tyr	Ser	Val	Trp
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 <212> PRT

<213> Artificial Sequence

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<223> synthetic

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Leu	Arg	Gly 35	Ala	Arg	Ala	Ser	Ala 40	Ala	Ala	Asp	Thr 45	Leu	Ser	Met	Arg
Thr	Ser 50	Ala	Arg	Ala	Ala 55	Pro	Arg	His	Gln	His 60	Gln	Gln	Ala	Arg	Arg
Gly 65	Ala	Arg	Phe	Pro 70	Ser	Leu	Val	Val	Cys	Ala 75	Ser	Ala	Gly	Ala	Met 80
Ala	Lys	Tyr	Leu 85	Glu	Leu	Glu	Glu	Gly	Gly 90	Val	Ile	Met	Gln	Ala 95	Phe
Tyr	Trp	Asp 100	Val	Pro	Ser	Gly	Gly 105	Ile	Trp	Trp	Asp	Thr 110	Ile	Arg	Gln
Lys	Ile 115	Pro	Glu	Trp	Tyr	Asp	Ala 120	Gly	Ile	Ser	Ala 125	Ile	Trp	Ile	Pro
Pro 130	Ala	Ser	Lys	Gly	Met 135	Ser	Gly	Gly	Tyr	Ser	Met 140	Gly	Tyr	Asp	Pro
Tyr 145	Asp	Tyr	Phe	Asp 150	Leu	Gly	Glu	Tyr	Tyr	Gln 155	Lys	Gly	Thr	Val	Glu 160
Thr	Arg	Phe	Gly 165	Ser	Lys	Gln	Glu	Leu	Ile 170	Asn	Met	Ile	Asn	Thr 175	Ala
His	Ala	Tyr 180	Gly	Ile	Lys	Val	Ile 185	Ala	Asp	Ile	Val 190	Ile	Asn	His	Arg
Ala	Gly 195	Gly	Asp	Leu	Glu	Trp 200	Asn	Pro	Phe	Val	Gly 205	Asp	Tyr	Thr	Trp
Thr	Asp 210	Phe	Ser	Lys	Val 215	Ala	Ser	Gly	Lys	Tyr	Thr 220	Ala	Asn	Tyr	Leu
Asp 225	Phe	His	Pro	Asn 230	Glu	Leu	His	Ala	Gly	Asp 235	Ser	Gly	Thr	Phe	Gly 240
Gly	Tyr	Pro	Asp 245	Ile	Cys	His	Asp	Lys	Ser 250	Trp	Asp	Gln	Tyr	Trp 255	Leu
Trp	Ala	Ser 260	Gln	Glu	Ser	Tyr	Ala 265	Ala	Tyr	Leu	Arg	Ser 270	Ile	Gly	Ile
Asp	Ala 275	Trp	Arg	Phe	Asp	Tyr 280	Val	Lys	Gly	Tyr	Gly 285	Ala	Trp	Val	Val
Lys	Asp 290	Trp	Leu	Asn 295	Trp	Trp	Gly	Gly	Trp	Ala 300	Val	Gly	Glu	Tyr	Trp
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Lys	Val	Phe	Asp 325	Phe	Pro	Leu	Tyr	Tyr	Lys 330	Met	Asp	Ala	Ala	Phe	Asp 335
Asn	Lys 340	Asn	Ile	Pro	Ala	Leu	Val 345	Glu	Ala	Leu	Lys	Asn 350	Gly	Gly	Thr
Val	Val 355	Ser	Arg	Asp	Pro	Phe	Lys 360	Ala	Val	Thr	Phe 365	Val	Ala	Asn	His
Asp	Thr 370	Asp	Ile	Ile	Trp	Asn 375	Lys	Tyr	Pro	Ala 380	Tyr	Ala	Phe	Ile	Leu



Thr	Tyr	Glu	Gly	Gln	Pro	Thr	Ile	Phe	Tyr	Arg	Asp	Tyr	Glu	Glu	Trp
385					390					395					400
Leu	Asn	Lys	Asp	Lys	Leu	Lys	Asn	Leu	Ile	Trp	Ile	His	Asp	Asn	Leu
				405					410					415	
Ala	Gly	Gly	Ser	Thr	Ser	Ile	Val	Tyr	Tyr	Asp	Ser	Asp	Glu	Met	Ile
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Phe	Val	Arg	Asn	Gly	Tyr	Gly	Ser	Lys	Pro	Gly	Leu	Ile	Thr	Tyr	Ile
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Asn	Leu	Gly	Ser	Ser	Lys	Val	Gly	Arg	Trp	Val	Tyr	Val	Pro	Lys	Phe
	450					455					460				
Ala	Gly	Ala	Cys	Ile	His	Glu	Tyr	Thr	Gly	Asn	Leu	Gly	Gly	Trp	Val
465					470					475					480
Asp	Lys	Tyr	Val	Tyr	Ser	Ser	Gly	Trp	Val	Tyr	Leu	Glu	Ala	Pro	Ala
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Tyr	Asp	Pro	Ala	Asn	Gly	Gln	Tyr	Gly	Tyr	Ser	Val	Trp	Ser	Tyr	Cys
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 <223> synthetic

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Thr	Ser	Ala	Arg	Ala	Ala	Pro	Arg	His	Gln	His	Gln	Gln	Ala	Arg	Arg
	50					55					60				
Gly	Ala	Arg	Phe	Pro	Ser	Leu	Val	Val	Cys	Ala	Ser	Ala	Gly	Ala	Met
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Ala	Lys	Tyr	Leu	Glu	Leu	Glu	Glu	Gly	Gly	Val	Ile	Met	Gln	Ala	Phe
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Tyr	Trp	Asp	Val	Pro	Ser	Gly	Gly	Ile	Trp	Trp	Asp	Thr	Ile	Arg	Gln
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Lys	Ile	Pro	Glu	Trp	Tyr	Asp	Ala	Gly	Ile	Ser	Ala	Ile	Trp	Ile	Pro
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Pro	Ala	Ser	Lys	Gly	Met	Ser	Gly	Gly	Tyr	Ser	Met	Gly	Tyr	Asp	Pro
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Tyr	Asp	Tyr	Phe	Asp	Leu	Gly	Glu	Tyr	Tyr	Gln	Lys	Gly	Thr	Val	Glu
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Thr	Arg	Phe	Gly	Ser	Lys	Gln	Glu	Leu	Ile	Asn	Met	Ile	Asn	Thr	Ala
			165					170						175	
His	Ala	Tyr	Gly	Ile	Lys	Val	Ile	Ala	Asp	Ile	Val	Ile	Asn	His	Arg
			180					185					190		
Ala	Gly	Gly	Asp	Leu	Glu	Trp	Asn	Pro	Phe	Val	Gly	Asp	Tyr	Thr	Trp

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Thr	Asp	Phe	Ser	Lys	Val	Ala	Ser	Gly	Lys	Tyr	Thr	Ala	Asn	Tyr	Leu
	210					215					220				
Asp	Phe	His	Pro	Asn	Glu	Leu	His	Ala	Gly	Asp	Ser	Gly	Thr	Phe	Gly
225					230					235					240
Gly	Tyr	Pro	Asp	Ile	Cys	His	Asp	Lys	Ser	Trp	Asp	Gln	Tyr	Trp	Leu
				245						250					255
Trp	Ala	Ser	Gln	Glu	Ser	Tyr	Ala	Ala	Tyr	Leu	Arg	Ser	Ile	Gly	Ile
			260					265					270		
Asp	Ala	Trp	Arg	Phe	Asp	Tyr	Val	Lys	Gly	Tyr	Gly	Ala	Trp	Val	Val
		275					280					285			
Lys	Asp	Trp	Leu	Asn	Trp	Trp	Gly	Gly	Trp	Ala	Val	Gly	Glu	Tyr	Trp
	290					295					300				
Asp	Thr	Asn	Val	Asp	Ala	Leu	Leu	Asn	Trp	Ala	Tyr	Ser	Ser	Gly	Ala
305					310					315					320
Lys	Val	Phe	Asp	Phe	Pro	Leu	Tyr	Tyr	Lys	Met	Asp	Ala	Ala	Phe	Asp
				325					330					335	
Asn	Lys	Asn	Ile	Pro	Ala	Leu	Val	Glu	Ala	Leu	Lys	Asn	Gly	Gly	Thr
			340					345					350		
Val	Val	Ser	Arg	Asp	Pro	Phe	Lys	Ala	Val	Thr	Phe	Val	Ala	Asn	His
		355					360					365			
Asp	Thr	Asp	Ile	Ile	Trp	Asn	Lys	Tyr	Pro	Ala	Tyr	Ala	Phe	Ile	Leu
	370					375					380				
Thr	Tyr	Glu	Gly	Gln	Pro	Thr	Ile	Phe	Tyr	Arg	Asp	Tyr	Glu	Glu	Trp
385					390					395					400
Leu	Asn	Lys	Asp	Lys	Leu	Lys	Asn	Leu	Ile	Trp	Ile	His	Asp	Asn	Leu
				405					410					415	
Ala	Gly	Gly	Ser	Thr	Ser	Ile	Val	Tyr	Tyr	Asp	Ser	Asp	Glu	Met	Ile
			420					425					430		
Phe	Val	Arg	Asn	Gly	Tyr	Gly	Ser	Lys	Pro	Gly	Leu	Ile	Thr	Tyr	Ile
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Asn	Leu	Gly	Ser	Ser	Lys	Val	Gly	Arg	Trp	Val	Tyr	Val	Pro	Lys	Phe
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Ala	Gly	Ala	Cys	Ile	His	Glu	Tyr	Thr	Gly	Asn	Leu	Gly	Gly	Trp	Val
465					470					475					480
Asp	Lys	Tyr	Val	Tyr	Ser	Ser	Gly	Trp	Val	Tyr	Leu	Glu	Ala	Pro	Ala
				485					490					495	
Tyr	Asp	Pro	Ala	Asn	Gly	Gln	Tyr	Gly	Tyr	Ser	Val	Trp	Ser	Tyr	Cys
		500						505					510		
Gly	Val	Gly	Thr	Ser	Ile	Ala	Gly	Ile	Leu	Glu	Ala	Asp	Arg	Val	Leu
		515					520					525			
Thr	Val	Ser	Pro	Tyr	Tyr	Ala	Glu	Glu	Leu	Ile	Ser	Gly	Ile	Ala	Arg
	530					535						540			
Gly	Cys	Glu	Leu	Asp	Asn	Ile	Met	Arg	Leu	Thr	Gly	Ile	Thr	Gly	Ile
545					550					555					560
Val	Asn	Gly	Met	Asp	Val	Ser	Glu	Trp	Asp	Pro	Ser	Arg	Asp	Lys	Tyr
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Ile	Ala	Val	Lys	Tyr	Asp	Val	Ser	Thr	Ala	Val	Glu	Ala	Lys	Ala	Leu
			580					585					590		
Asn	Lys	Glu	Ala	Leu	Gln	Ala	Glu	Val	Gly	Leu	Pro	Val	Asp	Arg	Asn
		595					600					605			
Ile	Pro	Leu	Val	Ala	Phe	Ile	Gly	Arg	Leu	Glu	Glu	Gln	Lys	Gly	Pro
	610					615					620				
Asp	Val	Met	Ala	Ala	Ala	Ile	Pro	Gln	Leu	Met	Glu	Met	Val	Glu	Asp

625					630						635					640
Val	Gln	Ile	Val	Leu	Leu	Gly	Thr	Gly	Lys	Lys	Lys	Phe	Glu	Arg	Met	
				645					650					655		
Leu	Met	Ser	Ala	Glu	Glu	Lys	Phe	Pro	Gly	Lys	Val	Arg	Ala	Val	Val	
			660					665					670			
Lys	Phe	Asn	Ala	Ala	Leu	Ala	His	His	Ile	Met	Ala	Gly	Ala	Asp	Val	
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Leu	Ala	Val	Thr	Ser	Arg	Phe	Glu	Pro	Cys	Gly	Leu	Ile	Gln	Leu	Gln	
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Gly	Met	Arg	Tyr	Gly	Thr	Pro	Cys	Ala	Cys	Ala	Ser	Thr	Gly	Gly	Leu	
705				710						715				720		
Val	Asp	Thr	Ile	Ile	Glu	Gly	Lys	Thr	Gly	Phe	His	Met	Gly	Arg	Leu	
			725					730					735			
Ser	Val	Asp	Cys	Asn	Val	Val	Glu	Pro	Ala	Asp	Val	Lys	Lys	Val	Ala	
		740					745			750						
Thr	Thr	Leu	Gln	Arg	Ala	Ile	Lys	Val	Val	Gly	Thr	Pro	Ala	Tyr	Glu	
	755					760				765						
Glu	Met	Val	Arg	Asn	Cys	Met	Ile	Gln	Asp	Leu	Ser	Trp	Lys	Gly	Pro	
	770				775					780						
Ala	Lys	Asn	Trp	Glu	Asn	Val	Leu	Leu	Ser	Leu	Gly	Val	Ala	Gly	Gly	
785				790						795				800		
Glu	Pro	Gly	Val	Glu	Gly	Glu	Glu	Ile	Ala	Pro	Leu	Ala	Lys	Glu	Asn	
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Val	Ala	Ala	Pro													
			820													

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 <213> Artificial Sequence

<220>  
 <223> synthetic

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 Ala Thr Ser

<210> 18  
 <211> 444  
 <212> PRT  
 <213> Thermotoga maritima

<400> 18  
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 Ile Asp Gly Lys Pro Leu Lys Asp His Leu Lys Phe Ser Val Ala Phe  
 35 40 45

Trp	His	Thr	Phe	Val	Asn	Glu	Gly	Arg	Asp	Pro	Phe	Gly	Asp	Pro	Thr
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Ala	Glu	Arg	Pro	Trp	Asn	Arg	Phe	Ser	Asp	Pro	Met	Asp	Lys	Ala	Phe
65					70				75						80
Ala	Arg	Val	Asp	Ala	Leu	Phe	Glu	Phe	Cys	Glu	Lys	Leu	Asn	Ile	Glu
				85					90					95	
Tyr	Phe	Cys	Phe	His	Asp	Arg	Asp	Ile	Ala	Pro	Glu	Gly	Lys	Thr	Leu
			100				105						110		
Arg	Glu	Thr	Asn	Lys	Ile	Leu	Asp	Lys	Val	Val	Glu	Arg	Ile	Lys	Glu
		115					120					125			
Arg	Met	Lys	Asp	Ser	Asn	Val	Lys	Leu	Leu	Trp	Gly	Thr	Ala	Asn	Leu
	130					135					140				
Phe	Ser	His	Pro	Arg	Tyr	Met	His	Gly	Ala	Ala	Thr	Thr	Cys	Ser	Ala
145					150				155						160
Asp	Val	Phe	Ala	Tyr	Ala	Ala	Ala	Gln	Val	Lys	Lys	Ala	Leu	Glu	Ile
			165					170						175	
Thr	Lys	Glu	Leu	Gly	Gly	Glu	Gly	Tyr	Val	Phe	Trp	Gly	Gly	Arg	Glu
			180					185					190		
Gly	Tyr	Glu	Thr	Leu	Leu	Asn	Thr	Asp	Leu	Gly	Leu	Glu	Leu	Glu	Asn
		195					200					205			
Leu	Ala	Arg	Phe	Leu	Arg	Met	Ala	Val	Glu	Tyr	Ala	Lys	Lys	Ile	Gly
	210					215					220				
Phe	Thr	Gly	Gln	Phe	Leu	Ile	Glu	Pro	Lys	Pro	Lys	Glu	Pro	Thr	Lys
225					230					235					240
His	Gln	Tyr	Asp	Phe	Asp	Val	Ala	Thr	Ala	Tyr	Ala	Phe	Leu	Lys	Asn
			245						250					255	
His	Gly	Leu	Asp	Glu	Tyr	Phe	Lys	Phe	Asn	Ile	Glu	Ala	Asn	His	Ala
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Thr	Leu	Ala	Gly	His	Thr	Phe	Gln	His	Glu	Leu	Arg	Met	Ala	Arg	Ile
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Leu	Gly	Lys	Leu	Gly	Ser	Ile	Asp	Ala	Asn	Gln	Gly	Asp	Leu	Leu	Leu
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Gly	Trp	Asp	Thr	Asp	Gln	Phe	Pro	Thr	Asn	Ile	Tyr	Asp	Thr	Thr	Leu
305					310					315					320
Ala	Met	Tyr	Glu	Val	Ile	Lys	Ala	Gly	Gly	Phe	Thr	Lys	Gly	Gly	Leu
			325						330					335	
Asn	Phe	Asp	Ala	Lys	Val	Arg	Arg	Ala	Ser	Tyr	Lys	Val	Glu	Asp	Leu
			340					345					350		
Phe	Ile	Gly	His	Ile	Ala	Gly	Met	Asp	Thr	Phe	Ala	Leu	Gly	Phe	Lys
	355						360					365			
Ile	Ala	Tyr	Lys	Leu	Ala	Lys	Asp	Gly	Val	Phe	Asp	Lys	Phe	Ile	Glu
	370					375					380				
Glu	Lys	Tyr	Arg	Ser	Phe	Lys	Glu	Gly	Ile	Gly	Lys	Glu	Ile	Val	Glu
385					390					395					400
Gly	Lys	Thr	Asp	Phe	Glu	Lys	Leu	Glu	Glu	Tyr	Ile	Ile	Asp	Lys	Glu
			405						410					415	
Asp	Ile	Glu	Leu	Pro	Ser	Gly	Lys	Gln	Glu	Tyr	Leu	Glu	Ser	Leu	Leu
			420					425					430		
Asn	Ser	Tyr	Ile	Val	Lys	Thr	Ile	Ala	Glu	Leu	Arg				
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&lt;210&gt; 19

&lt;211&gt; 1335

&lt;212&gt; DNA

<213> *Thermotoga maritima*

&lt;400&gt; 19

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cacctcaagt tctccgtggc cttctggcac accttcgtga acgagggccg cgacccgttc 180
ggcgacccga ccgccgagcg cccgtggaac cgcttctccg acccgatgga caaggccttc 240
gcccgcgtgg acgcccctct cgagttctgc gagaagctca acatcgagta cttctgcttc 300
cacgaccgcg acatcgcccc ggagggcaag accctccgcg agaccaacaa gatcctcgac 360
aaggtggtgg agcgcaccaa ggagcgcacg aaggactcca acgtgaagct cctctggggc 420
accgccaacc tcttctccca cccgcgctac atgcacggcg ccgccaccac ctgctccgcc 480
gacgtgttcg cctacgcccg cgcccaggtg aagaaggccc tggagatcac caaggagctg 540
ggcggcgagg gctacgtgtt ctggggcggc cgcgagggtt acgagaccct cctcaacacc 600
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gagtacttca agttcaacat cgaggccaac cacgccacc tgcgcggcca caccttccag 840
cacgagctgc gcatggcccg catcctcggc aagctcggct ccacgacgc caaccagggc 900
gacctcctcc tcggctggga caccgaccag ttcccgaaca acatctacga caccaccctc 960
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gacaccttcg cctcgggtt caagatcgcc tacaagctcg ccaaggacgg cgtgttcgac 1140
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gccgagctgc gctga                                     1335

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&lt;210&gt; 20

&lt;211&gt; 444

&lt;212&gt; PRT

<213> *Thermotoga neapolitana*

&lt;400&gt; 20

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          20           25           30
Ile Asp Gly Lys Pro Leu Lys Asp His Leu Lys Phe Ser Val Ala Phe
          35           40           45
Trp His Thr Phe Val Asn Glu Gly Arg Asp Pro Phe Gly Asp Pro Thr
          50           55           60
Ala Asp Arg Pro Trp Asn Arg Tyr Thr Asp Pro Met Asp Lys Ala Phe
          65           70           75           80
Ala Arg Val Asp Ala Leu Phe Glu Phe Cys Glu Lys Leu Asn Ile Glu
          85           90           95
Tyr Phe Cys Phe His Asp Arg Asp Ile Ala Pro Glu Gly Lys Thr Leu
          100          105          110
Arg Glu Thr Asn Lys Ile Leu Asp Lys Val Val Glu Arg Ile Lys Glu
          115          120          125
Arg Met Lys Asp Ser Asn Val Lys Leu Leu Trp Gly Thr Ala Asn Leu
          130          135          140
Phe Ser His Pro Arg Tyr Met His Gly Ala Ala Thr Thr Cys Ser Ala
          145          150          155          160

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Asp Val Phe Ala Tyr Ala Ala Ala Gln Val Lys Lys Ala Leu Glu Ile  
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 Thr Lys Glu Leu Gly Gly Glu Gly Tyr Val Phe Trp Gly Gly Arg Glu  
 180 185 190  
 Gly Tyr Glu Thr Leu Leu Asn Thr Asp Leu Gly Phe Glu Leu Glu Asn  
 195 200 205  
 Leu Ala Arg Phe Leu Arg Met Ala Val Asp Tyr Ala Lys Arg Ile Gly  
 210 215 220  
 Phe Thr Gly Gln Phe Leu Ile Glu Pro Lys Pro Lys Glu Pro Thr Lys  
 225 230 235 240  
 His Gln Tyr Asp Phe Asp Val Ala Thr Ala Tyr Ala Phe Leu Lys Ser  
 245 250 255  
 His Gly Leu Asp Glu Tyr Phe Lys Phe Asn Ile Glu Ala Asn His Ala  
 260 265 270  
 Thr Leu Ala Gly His Thr Phe Gln His Glu Leu Arg Met Ala Arg Ile  
 275 280 285  
 Leu Gly Lys Leu Gly Ser Ile Asp Ala Asn Gln Gly Asp Leu Leu Leu  
 290 295 300  
 Gly Trp Asp Thr Asp Gln Phe Pro Thr Asn Val Tyr Asp Thr Thr Leu  
 305 310 315 320  
 Ala Met Tyr Glu Val Ile Lys Ala Gly Gly Phe Thr Lys Gly Gly Leu  
 325 330 335  
 Asn Phe Asp Ala Lys Val Arg Arg Ala Ser Tyr Lys Val Glu Asp Leu  
 340 345 350  
 Phe Ile Gly His Ile Ala Gly Met Asp Thr Phe Ala Leu Gly Phe Lys  
 355 360 365  
 Val Ala Tyr Lys Leu Val Lys Asp Gly Val Leu Asp Lys Phe Ile Glu  
 370 375 380  
 Glu Lys Tyr Arg Ser Phe Arg Glu Gly Ile Gly Arg Asp Ile Val Glu  
 385 390 395 400  
 Gly Lys Val Asp Phe Glu Lys Leu Glu Glu Tyr Ile Ile Asp Lys Glu  
 405 410 415  
 Thr Ile Glu Leu Pro Ser Gly Lys Gln Glu Tyr Leu Glu Ser Leu Ile  
 420 425 430  
 Asn Ser Tyr Ile Val Lys Thr Ile Leu Glu Leu Arg  
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&lt;210&gt; 21

&lt;211&gt; 1335

&lt;212&gt; DNA

&lt;213&gt; Thermotoga neapolitana

&lt;400&gt; 21

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 cacctcaagt tctccgtggc cttctggcac accttcgtga acgagggccg cgaccggttc 180  
 ggcgacccga ccgcccagcc cccgtggaac cgctacaccg acccgatgga caaggccttc 240  
 gccgcgtgg acgccttctt cgagttctgc gagaagctca acatcgagta cttctgcttc 300  
 cacgaccgcg acatcgcccc ggagggaag accctccgcg agaccaacaa gatcctcgac 360  
 aagggtggtg agcgcacaa ggagcgcacg aaggactcca acgtgaagct cctctggggc 420  
 accgccaacc tcttctccca cccgcgctac atgcacggcg ccgccaccac ctgctccgcc 480  
 gacgtgttcg cctacgcgcg cgcccaggtg aagaaggccc tggagatcac caaggagctg 540  
 ggcggcgagg gctacgtgtt ctggggcggc cgcgagggtt acgagaccct cctcaacacc 600

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gacctcggct tcgagctgga gaacctcgcc cgcttcctcc gcatggccgt ggactacgcc 660
aagcgcatcg gcttcaccgg ccagttcctc atcgagccga agccgaagga gccgaccaag 720
caccagtacg acttcgacgt ggccaccgcc tacgccttcc tcaagtccca cggcctcgac 780
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gacctcctcc tcggctggga caccgaccag ttcccgaaca acgtgtacga caccaccctc 960
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gacaccttcg ccctcggctt caaggtggcc tacaagctcg tgaaggacgg cgtgctcgac 1140
aagttcatcg aggagaagta ccgctccttc cgcgagggca tcggccgcga catcgtggag 1200
ggcaaggtgg acttcgagaa gctggaggag tacatcatcg acaaggagac catcgagctg 1260
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<400> 22  
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<220>  
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<400> 23  
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<210> 24  
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 <212> PRT  
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<220>  
 <223> synthetic

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 Thr Gly Glu Asp Asp Phe Gly Lys Val Ala Val Val Lys Leu Pro Met  
 35 40 45  
 Asp Leu Thr Lys Val Gly Ile Ile Val Arg Leu Asn Glu Trp Gln Ala  
 50 55 60  
 Lys Asp Val Ala Lys Asp Arg Phe Ile Glu Ile Lys Asp Gly Lys Ala

65					70					75					80
Glu	Val	Trp	Ile	Leu	Gln	Gly	Val	Glu	Glu	Ile	Phe	Tyr	Glu	Lys	Pro
				85					90					95	
Asp	Thr	Ser	Pro	Arg	Ile	Phe	Phe	Ala	Gln	Ala	Arg	Ser	Asn	Lys	Val
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Ile	Glu	Ala	Phe	Leu	Thr	Asn	Pro	Val	Asp	Thr	Lys	Lys	Lys	Glu	Leu
		115					120					125			
Phe	Lys	Val	Thr	Val	Asp	Gly	Lys	Glu	Ile	Pro	Val	Ser	Arg	Val	Glu
	130					135				140					
Lys	Ala	Asp	Pro	Thr	Asp	Ile	Asp	Val	Thr	Asn	Tyr	Val	Arg	Ile	Val
145					150					155					160
Leu	Ser	Glu	Ser	Leu	Lys	Glu	Glu	Asp	Leu	Arg	Lys	Asp	Val	Glu	Leu
				165					170					175	
Ile	Ile	Glu	Gly	Tyr	Lys	Pro	Ala	Arg	Val	Ile	Met	Met	Glu	Ile	Leu
			180					185					190		
Asp	Asp	Tyr	Tyr	Tyr	Asp	Gly	Glu	Leu	Gly	Ala	Val	Tyr	Ser	Pro	Glu
		195					200					205			
Lys	Thr	Ile	Phe	Arg	Val	Trp	Ser	Pro	Val	Ser	Lys	Trp	Val	Lys	Val
	210					215					220				
Leu	Leu	Phe	Lys	Asn	Gly	Glu	Asp	Thr	Glu	Pro	Tyr	Gln	Val	Val	Asn
225					230					235					240
Met	Glu	Tyr	Lys	Gly	Asn	Gly	Val	Trp	Glu	Ala	Val	Val	Glu	Gly	Asp
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Leu	Asp	Gly	Val	Phe	Tyr	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Gly	Lys	Ile
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Arg	Thr	Thr	Val	Asp	Pro	Tyr	Ser	Lys	Ala	Val	Tyr	Ala	Asn	Asn	Gln
		275					280					285			
Glu	Ser	Ala	Val	Val	Asn	Leu	Ala	Arg	Thr	Asn	Pro	Glu	Gly	Trp	Glu
	290					295					300				
Asn	Asp	Arg	Gly	Pro	Lys	Ile	Glu	Gly	Tyr	Glu	Asp	Ala	Ile	Ile	Tyr
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Glu	Ile	His	Ile	Ala	Asp	Ile	Thr	Gly	Leu	Glu	Asn	Ser	Gly	Val	Lys
				325					330					335	
Asn	Lys	Gly	Leu	Tyr	Leu	Gly	Leu	Thr	Glu	Glu	Asn	Thr	Lys	Ala	Pro
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		355					360					365			
His	Val	His	Ile	Leu	Pro	Phe	Phe	Asp	Phe	Tyr	Thr	Gly	Asp	Glu	Leu
	370					375					380				
Asp	Lys	Asp	Phe	Glu	Lys	Tyr	Tyr	Asn	Trp	Gly	Tyr	Asp	Pro	Tyr	Leu
385					390					395					400
Phe	Met	Val	Pro	Glu	Gly	Arg	Tyr	Ser	Thr	Asp	Pro	Lys	Asn	Pro	His
				405					410					415	
Thr	Arg	Ile	Arg	Glu	Val	Lys	Glu	Met	Val	Lys	Ala	Leu	His	Lys	His
			420					425					430		
Gly	Ile	Gly	Val	Ile	Met	Asp	Met	Val	Phe	Pro	His	Thr	Tyr	Gly	Ile
		435					440					445			
Gly	Glu	Leu	Ser	Ala	Phe	Asp	Gln	Thr	Val	Pro	Tyr	Tyr	Phe	Tyr	Arg
	450					455					460				
Ile	Asp	Lys	Thr	Gly	Ala	Tyr	Leu	Asn	Glu	Ser	Gly	Cys	Gly	Asn	Val
465					470					475					480
Ile	Ala	Ser	Glu	Arg	Pro	Met	Met	Arg	Lys	Phe	Ile	Val	Asp	Thr	Val
				485					490					495	
Thr	Tyr	Trp	Val	Lys	Glu	Tyr	His	Ile	Asp	Gly	Phe	Arg	Phe	Asp	Gln

			500					505				510					
Met	Gly	Leu	Ile	Asp	Lys	Lys	Thr	Met	Leu	Glu	Val	Glu	Arg	Ala	Leu		
		515					520					525					
His	Lys	Ile	Asp	Pro	Thr	Ile	Ile	Leu	Tyr	Gly	Glu	Pro	Trp	Gly	Gly		
	530					535					540						
Trp	Gly	Ala	Pro	Ile	Arg	Phe	Gly	Lys	Ser	Asp	Val	Ala	Gly	Thr	His		
545					550					555					560		
Val	Ala	Ala	Phe	Asn	Asp	Glu	Phe	Arg	Asp	Ala	Ile	Arg	Gly	Ser	Val		
			565				570							575			
Phe	Asn	Pro	Ser	Val	Lys	Gly	Phe	Val	Met	Gly	Gly	Tyr	Gly	Lys	Glu		
		580					585						590				
Thr	Lys	Ile	Lys	Arg	Gly	Val	Val	Gly	Ser	Ile	Asn	Tyr	Asp	Gly	Lys		
	595					600						605					
Leu	Ile	Lys	Ser	Phe	Ala	Leu	Asp	Pro	Glu	Glu	Thr	Ile	Asn	Tyr	Ala		
	610					615					620						
Ala	Cys	His	Asp	Asn	His	Thr	Leu	Trp	Asp	Lys	Asn	Tyr	Leu	Ala	Ala		
625				630						635					640		
Lys	Ala	Asp	Lys	Lys	Lys	Glu	Trp	Thr	Glu	Glu	Glu	Leu	Lys	Asn	Ala		
		645						650						655			
Gln	Lys	Leu	Ala	Gly	Ala	Ile	Leu	Leu	Thr	Ser	Gln	Gly	Val	Pro	Phe		
	660						665					670					
Leu	His	Gly	Gly	Gln	Asp	Phe	Cys	Arg	Thr	Thr	Asn	Phe	Asn	Asp	Asn		
	675					680					685						
Ser	Tyr	Asn	Ala	Pro	Ile	Ser	Ile	Asn	Gly	Phe	Asp	Tyr	Glu	Arg	Lys		
	690				695						700						
Leu	Gln	Phe	Ile	Asp	Val	Phe	Asn	Tyr	His	Lys	Gly	Leu	Ile	Lys	Leu		
705				710						715					720		
Arg	Lys	Glu	His	Pro	Ala	Phe	Arg	Leu	Lys	Asn	Ala	Glu	Glu	Ile	Lys		
		725						730						735			
Lys	His	Leu	Glu	Phe	Leu	Pro	Gly	Gly	Arg	Arg	Ile	Val	Ala	Phe	Met		
	740						745					750					
Leu	Lys	Asp	His	Ala	Gly	Gly	Asp	Pro	Trp	Lys	Asp	Ile	Val	Val	Ile		
	755					760					765						
Tyr	Asn	Gly	Asn	Leu	Glu	Lys	Thr	Thr	Tyr	Lys	Leu	Pro	Glu	Gly	Lys		
	770				775						780						
Trp	Asn	Val	Val	Val	Asn	Ser	Gln	Lys	Ala	Gly	Thr	Glu	Val	Ile	Glu		
785				790						795					800		
Thr	Val	Glu	Gly	Thr	Ile	Glu	Leu	Asp	Pro	Leu	Ser	Ala	Tyr	Val	Leu		
		805						810						815			
Tyr	Arg	Glu	Ser	Glu	Lys	Asp	Glu	Leu									
		820					825										

&lt;210&gt; 25

&lt;211&gt; 2478

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 25

atgagggtgt tgctcgttgc cctcgctctc ctggctctcg ctgcgagcgc caccagcgct 60  
 ggccactggt acaagcacca gcgcgcctac cagttcaccg gcgaggacga cttcggaag 120

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gtggccgtgg tgaagctccc gatggacctc accaaggtgg gcatcatcgt ggcctcaac 180
gagtggcagg cgaaggacgt ggccaaggac cgcttcacgc agatcaagga cggcaaggcc 240
gaggtgtgga tactccaggg cgtggaggag atcttctacg agaagccgga cacctccccg 300
cgcatcttct tcgcccaggc ccgctccaac aaggtgatcg aggccttcct caccaaccgc 360
gtggacacca agaagaagga gctgttcaag gtgaccgtcg acggcaagga gatcccggtg 420
tcccgcgtgg agaaggccga cccgaccgac atcgacgtga ccaactacgt ggcacatcgt 480
ctctccgagt ccctcaagga ggaggacctc cgcaaggacg tggagctgat catcgagggc 540
tacaagccgg cccgcgtgat catgatggag atcctcgacg actactacta cgacggcgag 600
ctggggggcg tgtactcccc ggagaagacc atcttccgcg tgtgggtccc ggtgtccaag 660
tgggtgaagg tgctcctctt caagaacggc gaggacaccg agccgtacca ggtggtgaac 720
atggagtaca agggcaacgg cgtgtgggag gccgtggtgg agggcgacct cgacggcggtg 780
ttctacctct accagctgga gaactacggc aagatccgca ccaccgtgga cccgtactcc 840
aaggccgtgt acgccaacaa ccaggagtct gcagtgggtg acctcgcccc caccaaccgc 900
gagggctggg agaacgaccg cggcccgaag atcgagggtc acgaggacgc catcatctac 960
gagatccaca tcgccgacat caccggcctg gagaactccg gcgtgaagaa caagggcctc 1020
tacctcggcc tcaccgagga gaacaccaag gccccggggc gcgtgaccac cggcctctcc 1080
cacctcgtgg agctggggcg gacccacgtg cacatcctcc cgttcttcga cttctacacc 1140
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ttcatggtgc cggagggccg ctactccacc gaccgaaga acccgcacac ccgaattcgc 1260
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gtgttccccg acacctacgg catcggcgag ctgtccgcct tcgaccagac cgtgccgtac 1380
tacttctacc gcatcgacaa gaccggcgcc tacctcaacg agtccggctg cggcaacgtg 1440
atcgctcccg agcgcccgat gatgcgcaag ttcacgtggt acaccgtgac ctactgggtg 1500
aaggagtacc acatcgacgg cttccgcttc gaccagatgg gcctcatcga caagaagacc 1560
atgctggagg tggagcgcg cctccacaag atcgaccgca ccatcatcct ctacggcgag 1620
ccgtggggcg gctggggggc cccgatccgc ttcggcaagt ccgacgtggc cggcaccac 1680
gtggccgcct tcaacgacga gttccgcgac gccatccgcg gctccgtgtt caaccgtcc 1740
gtgaagggtc tcgtgatggg cggctacggc aaggagacca agatcaagcg cggcgtggtg 1800
ggctccatca actacgacgg caagctcatc aagtccttcg ccctcgaccg ggaggagacc 1860
atcaactacg ccgcctgcca cgacaaccac accctctggg acaagaacta cctcgccgcc 1920
aaggccgaca agaagaagga gtggaccgag gaggagctga agaagccca gaagctcgcc 1980
ggcgccatcc tcctcactag tcagggcgtg ccgttcctcc acggcgggca ggacttctgc 2040
cgccaccaca acttcaacga caactcctac aacgccccga tctccatcaa cggcttcgac 2100
tacgagcgca agctccagtt catcgacgtg ttcaactacc acaaggcct catcaagctc 2160
cgcaaggagc acccggcctt ccgcctcaag aacgcgaggg agatcaagaa gcacctggag 2220
ttcctcccgg gcgggcgccc catcgtggcc ttcattgctc agaccacgc cggcgggcgc 2280
ccgtggaagg acatcgtggt gatctacaac ggcaacctgg agaagaccac ctacaagctc 2340
ccggagggca agtggaacgt ggtggtgaac tcccagaagg ccggcaccga ggtgatcgag 2400
accgtggagg gcaccatcga gctggacccg ctctccgcct acgtgctcta ccgcgagtc 2460
gagaaggacg agctgtga

```

<210> 26  
 <211> 718  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 26  
 Met Arg Val Leu Leu Val Ala Leu Ala Leu Leu Ala Leu Ala Ser  
 1 5 10 15  
 Ala Thr Ser Met Glu Thr Ile Lys Ile Tyr Glu Asn Lys Gly Val Tyr  
 20 25 30



Lys	Val	Val	Ile	Gly	Glu	Pro	Phe	Pro	Pro	Ile	Glu	Phe	Pro	Leu	Glu
		35					40					45			
Gln	Lys	Ile	Ser	Ser	Asn	Lys	Ser	Leu	Ser	Glu	Leu	Gly	Leu	Thr	Ile
	50					55					60				
Val	Gln	Gln	Gly	Asn	Lys	Val	Ile	Val	Glu	Lys	Ser	Leu	Asp	Leu	Lys
65					70					75					80
Glu	His	Ile	Ile	Gly	Leu	Gly	Glu	Lys	Ala	Phe	Glu	Leu	Asp	Arg	Lys
				85					90					95	
Arg	Lys	Arg	Tyr	Val	Met	Tyr	Asn	Val	Asp	Ala	Gly	Ala	Tyr	Lys	Lys
			100					105					110		
Tyr	Gln	Asp	Pro	Leu	Tyr	Val	Ser	Ile	Pro	Leu	Phe	Ile	Ser	Val	Lys
		115					120					125			
Asp	Gly	Val	Ala	Thr	Gly	Tyr	Phe	Phe	Asn	Ser	Ala	Ser	Lys	Val	Ile
	130					135					140				
Phe	Asp	Val	Gly	Leu	Glu	Glu	Tyr	Asp	Lys	Val	Ile	Val	Thr	Ile	Pro
145					150					155					160
Glu	Asp	Ser	Val	Glu	Phe	Tyr	Val	Ile	Glu	Gly	Pro	Arg	Ile	Glu	Asp
				165					170					175	
Val	Leu	Glu	Lys	Tyr	Thr	Glu	Leu	Thr	Gly	Lys	Pro	Phe	Leu	Pro	Pro
			180					185					190		
Met	Trp	Ala	Phe	Gly	Tyr	Met	Ile	Ser	Arg	Tyr	Ser	Tyr	Tyr	Pro	Gln
		195					200					205			
Asp	Lys	Val	Val	Glu	Leu	Val	Asp	Ile	Met	Gln	Lys	Glu	Gly	Phe	Arg
	210					215					220				
Val	Ala	Gly	Val	Phe	Leu	Asp	Ile	His	Tyr	Met	Asp	Ser	Tyr	Lys	Leu
225					230					235					240
Phe	Thr	Trp	His	Pro	Tyr	Arg	Phe	Pro	Glu	Pro	Lys	Lys	Leu	Ile	Asp
				245					250					255	
Glu	Leu	His	Lys	Arg	Asn	Val	Lys	Leu	Ile	Thr	Ile	Val	Asp	His	Gly
			260					265					270		
Ile	Arg	Val	Asp	Gln	Asn	Tyr	Ser	Pro	Phe	Leu	Ser	Gly	Met	Gly	Lys
		275					280					285			
Phe	Cys	Glu	Ile	Glu	Ser	Gly	Glu	Leu	Phe	Val	Gly	Lys	Met	Trp	Pro
	290					295					300				
Gly	Thr	Thr	Val	Tyr	Pro	Asp	Phe	Phe	Arg	Glu	Asp	Thr	Arg	Glu	Trp
305					310					315					320
Trp	Ala	Gly	Leu	Ile	Ser	Glu	Trp	Leu	Ser	Gln	Gly	Val	Asp	Gly	Ile
				325					330					335	
Trp	Leu	Asp	Met	Asn	Glu	Pro	Thr	Asp	Phe	Ser	Arg	Ala	Ile	Glu	Ile
			340					345					350		
Arg	Asp	Val	Leu	Ser	Ser	Leu	Pro	Val	Gln	Phe	Arg	Asp	Asp	Arg	Leu
			355				360					365			
Val	Thr	Thr	Phe	Pro	Asp	Asn	Val	Val	His	Tyr	Leu	Arg	Gly	Lys	Arg
						375					380				
Val	Lys	His	Glu	Lys	Val	Arg	Asn	Ala	Tyr	Pro	Leu	Tyr	Glu	Ala	Met
385					390					395					400
Ala	Thr	Phe	Lys	Gly	Phe	Arg	Thr	Ser	His	Arg	Asn	Glu	Ile	Phe	Ile
				405					410					415	
Leu	Ser	Arg	Ala	Gly	Tyr	Ala	Gly	Ile	Gln	Arg	Tyr	Ala	Phe	Ile	Trp
			420					425					430		
Thr	Gly	Asp	Asn	Thr	Pro	Ser	Trp	Asp	Asp	Leu	Lys	Leu	Gln	Leu	Gln
		435					440					445			
Leu	Val	Leu	Gly	Leu	Ser	Ile	Ser	Gly	Val	Pro	Phe	Val	Gly	Cys	Asp
	450					455					460				

```

Ile Gly Gly Phe Gln Gly Arg Asn Phe Ala Glu Ile Asp Asn Ser Met
465                               470                               475                               480
Asp Leu Leu Val Lys Tyr Tyr Ala Leu Ala Leu Phe Phe Pro Phe Tyr
                               485                               490                               495
Arg Ser His Lys Ala Thr Asp Gly Ile Asp Thr Glu Pro Val Phe Leu
                               500                               505                               510
Pro Asp Tyr Tyr Lys Glu Lys Val Lys Glu Ile Val Glu Leu Arg Tyr
                               515                               520                               525
Lys Phe Leu Pro Tyr Ile Tyr Ser Leu Ala Leu Glu Ala Ser Glu Lys
                               530                               535                               540
Gly His Pro Val Ile Arg Pro Leu Phe Tyr Glu Phe Gln Asp Asp Asp
545                               550                               555                               560
Asp Met Tyr Arg Ile Glu Asp Glu Tyr Met Val Gly Lys Tyr Leu Leu
                               565                               570                               575
Tyr Ala Pro Ile Val Ser Lys Glu Glu Ser Arg Leu Val Thr Leu Pro
                               580                               585                               590
Arg Gly Lys Trp Tyr Asn Tyr Trp Asn Gly Glu Ile Ile Asn Gly Lys
                               595                               600                               605
Ser Val Val Lys Ser Thr His Glu Leu Pro Ile Tyr Leu Arg Glu Gly
                               610                               615                               620
Ser Ile Ile Pro Leu Glu Gly Asp Glu Leu Ile Val Tyr Gly Glu Thr
625                               630                               635                               640
Ser Phe Lys Arg Tyr Asp Asn Ala Glu Ile Thr Ser Ser Ser Asn Glu
                               645                               650                               655
Ile Lys Phe Ser Arg Glu Ile Tyr Val Ser Lys Leu Thr Ile Thr Ser
                               660                               665                               670
Glu Lys Pro Val Ser Lys Ile Ile Val Asp Asp Ser Lys Glu Ile Gln
                               675                               680                               685
Val Glu Lys Thr Met Gln Asn Thr Tyr Val Ala Lys Ile Asn Gln Lys
690                               695                               700
Ile Arg Gly Lys Ile Asn Leu Glu Ser Glu Lys Asp Glu Leu
705                               710                               715

```

&lt;210&gt; 27

&lt;211&gt; 712

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 27

```

Met Arg Val Leu Leu Val Ala Leu Ala Leu Leu Ala Leu Ala Ala Ser
1                               5                               10                               15
Ala Thr Ser Met Glu Thr Ile Lys Ile Tyr Glu Asn Lys Gly Val Tyr
                               20                               25                               30
Lys Val Val Ile Gly Glu Pro Phe Pro Pro Ile Glu Phe Pro Leu Glu
                               35                               40                               45
Gln Lys Ile Ser Ser Asn Lys Ser Leu Ser Glu Leu Gly Leu Thr Ile
50                               55                               60
Val Gln Gln Gly Asn Lys Val Ile Val Glu Lys Ser Leu Asp Leu Lys
65                               70                               75                               80
Glu His Ile Ile Gly Leu Gly Glu Lys Ala Phe Glu Leu Asp Arg Lys

```

85						90						95					
Arg	Lys	Arg	Tyr	Val	Met	Tyr	Asn	Val	Asp	Ala	Gly	Ala	Tyr	Lys	Lys		
			100					105					110				
Tyr	Gln	Asp	Pro	Leu	Tyr	Val	Ser	Ile	Pro	Leu	Phe	Ile	Ser	Val	Lys		
		115					120					125					
Asp	Gly	Val	Ala	Thr	Gly	Tyr	Phe	Phe	Asn	Ser	Ala	Ser	Lys	Val	Ile		
	130					135					140						
Phe	Asp	Val	Gly	Leu	Glu	Glu	Tyr	Asp	Lys	Val	Ile	Val	Thr	Ile	Pro		
145					150					155					160		
Glu	Asp	Ser	Val	Glu	Phe	Tyr	Val	Ile	Glu	Gly	Pro	Arg	Ile	Glu	Asp		
				165					170					175			
Val	Leu	Glu	Lys	Tyr	Thr	Glu	Leu	Thr	Gly	Lys	Pro	Phe	Leu	Pro	Pro		
			180					185					190				
Met	Trp	Ala	Phe	Gly	Tyr	Met	Ile	Ser	Arg	Tyr	Ser	Tyr	Tyr	Pro	Gln		
		195					200					205					
Asp	Lys	Val	Val	Glu	Leu	Val	Asp	Ile	Met	Gln	Lys	Glu	Gly	Phe	Arg		
	210					215					220						
Val	Ala	Gly	Val	Phe	Leu	Asp	Ile	His	Tyr	Met	Asp	Ser	Tyr	Lys	Leu		
225					230					235					240		
Phe	Thr	Trp	His	Pro	Tyr	Arg	Phe	Pro	Glu	Pro	Lys	Lys	Leu	Ile	Asp		
				245					250					255			
Glu	Leu	His	Lys	Arg	Asn	Val	Lys	Leu	Ile	Thr	Ile	Val	Asp	His	Gly		
			260					265					270				
Ile	Arg	Val	Asp	Gln	Asn	Tyr	Ser	Pro	Phe	Leu	Ser	Gly	Met	Gly	Lys		
		275					280					285					
Phe	Cys	Glu	Ile	Glu	Ser	Gly	Glu	Leu	Phe	Val	Gly	Lys	Met	Trp	Pro		
	290					295					300						
Gly	Thr	Thr	Val	Tyr	Pro	Asp	Phe	Phe	Arg	Glu	Asp	Thr	Arg	Glu	Trp		
305					310					315					320		
Trp	Ala	Gly	Leu	Ile	Ser	Glu	Trp	Leu	Ser	Gln	Gly	Val	Asp	Gly	Ile		
				325					330					335			
Trp	Leu	Asp	Met	Asn	Glu	Pro	Thr	Asp	Phe	Ser	Arg	Ala	Ile	Glu	Ile		
			340					345					350				
Arg	Asp	Val	Leu	Ser	Ser	Leu	Pro	Val	Gln	Phe	Arg	Asp	Asp	Arg	Leu		
		355					360					365					
Val	Thr	Thr	Phe	Pro	Asp	Asn	Val	Val	His	Tyr	Leu	Arg	Gly	Lys	Arg		
		370				375					380						
Val	Lys	His	Glu	Lys	Val	Arg	Asn	Ala	Tyr	Pro	Leu	Tyr	Glu	Ala	Met		
385					390					395					400		
Ala	Thr	Phe	Lys	Gly	Phe	Arg	Thr	Ser	His	Arg	Asn	Glu	Ile	Phe	Ile		
				405					410					415			
Leu	Ser	Arg	Ala	Gly	Tyr	Ala	Gly	Ile	Gln	Arg	Tyr	Ala	Phe	Ile	Trp		
			420				425						430				
Thr	Gly	Asp	Asn	Thr	Pro	Ser	Trp	Asp	Asp	Leu	Lys	Leu	Gln	Leu	Gln		
		435					440					445					
Leu	Val	Leu	Gly	Leu	Ser	Ile	Ser	Gly	Val	Pro	Phe	Val	Gly	Cys	Asp		
	450					455					460						
Ile	Gly	Gly	Phe	Gln	Gly	Arg	Asn	Phe	Ala	Glu	Ile	Asp	Asn	Ser	Met		
465					470					475					480		
Asp	Leu	Leu	Val	Lys	Tyr	Tyr	Ala	Leu	Ala	Leu	Phe	Phe	Pro	Phe	Tyr		
				485					490					495			
Arg	Ser	His	Lys	Ala	Thr	Asp	Gly	Ile	Asp	Thr	Glu	Pro	Val	Phe	Leu		
			500					505					510				
Pro	Asp	Tyr	Tyr	Lys	Glu	Lys	Val	Lys	Glu	Ile	Val	Glu	Leu	Arg	Tyr		

```

      515      520      525
Lys Phe Leu Pro Tyr Ile Tyr Ser Leu Ala Leu Glu Ala Ser Glu Lys
      530      535      540
Gly His Pro Val Ile Arg Pro Leu Phe Tyr Glu Phe Gln Asp Asp Asp
545      550      555      560
Asp Met Tyr Arg Ile Glu Asp Glu Tyr Met Val Gly Lys Tyr Leu Leu
      565      570      575
Tyr Ala Pro Ile Val Ser Lys Glu Glu Ser Arg Leu Val Thr Leu Pro
      580      585      590
Arg Gly Lys Trp Tyr Asn Tyr Trp Asn Gly Glu Ile Ile Asn Gly Lys
      595      600      605
Ser Val Val Lys Ser Thr His Glu Leu Pro Ile Tyr Leu Arg Glu Gly
      610      615      620
Ser Ile Ile Pro Leu Glu Gly Asp Glu Leu Ile Val Tyr Gly Glu Thr
625      630      635      640
Ser Phe Lys Arg Tyr Asp Asn Ala Glu Ile Thr Ser Ser Ser Asn Glu
      645      650      655
Ile Lys Phe Ser Arg Glu Ile Tyr Val Ser Lys Leu Thr Ile Thr Ser
      660      665      670
Glu Lys Pro Val Ser Lys Ile Ile Val Asp Asp Ser Lys Glu Ile Gln
      675      680      685
Val Glu Lys Thr Met Gln Asn Thr Tyr Val Ala Lys Ile Asn Gln Lys
      690      695      700
Ile Arg Gly Lys Ile Asn Leu Glu
705      710

```

&lt;210&gt; 28

&lt;211&gt; 469

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 28

```

Met Arg Val Leu Leu Val Ala Leu Ala Leu Leu Ala Leu Ala Ala Ser
 1      5      10      15
Ala Thr Ser Met Ala Glu Phe Phe Pro Glu Ile Pro Lys Ile Gln Phe
      20      25      30
Glu Gly Lys Glu Ser Thr Asn Pro Leu Ala Phe Arg Phe Tyr Asp Pro
      35      40      45
Asn Glu Val Ile Asp Gly Lys Pro Leu Lys Asp His Leu Lys Phe Ser
      50      55      60
Val Ala Phe Trp His Thr Phe Val Asn Glu Gly Arg Asp Pro Phe Gly
65      70      75      80
Asp Pro Thr Ala Glu Arg Pro Trp Asn Arg Phe Ser Asp Pro Met Asp
      85      90      95
Lys Ala Phe Ala Arg Val Asp Ala Leu Phe Glu Phe Cys Glu Lys Leu
      100      105      110
Asn Ile Glu Tyr Phe Cys Phe His Asp Arg Asp Ile Ala Pro Glu Gly
      115      120      125
Lys Thr Leu Arg Glu Thr Asn Lys Ile Leu Asp Lys Val Val Glu Arg
      130      135      140

```

```

Ile Lys Glu Arg Met Lys Asp Ser Asn Val Lys Leu Leu Trp Gly Thr
145      150      155      160
Ala Asn Leu Phe Ser His Pro Arg Tyr Met His Gly Ala Ala Thr Thr
      165      170      175
Cys Ser Ala Asp Val Phe Ala Tyr Ala Ala Ala Gln Val Lys Lys Ala
      180      185      190
Leu Glu Ile Thr Lys Glu Leu Gly Gly Glu Gly Tyr Val Phe Trp Gly
      195      200      205
Gly Arg Glu Gly Tyr Glu Thr Leu Leu Asn Thr Asp Leu Gly Leu Glu
      210      215      220
Leu Glu Asn Leu Ala Arg Phe Leu Arg Met Ala Val Glu Tyr Ala Lys
225      230      235      240
Lys Ile Gly Phe Thr Gly Gln Phe Leu Ile Glu Pro Lys Pro Lys Glu
      245      250      255
Pro Thr Lys His Gln Tyr Asp Phe Asp Val Ala Thr Ala Tyr Ala Phe
      260      265      270
Leu Lys Asn His Gly Leu Asp Glu Tyr Phe Lys Phe Asn Ile Glu Ala
      275      280      285
Asn His Ala Thr Leu Ala Gly His Thr Phe Gln His Glu Leu Arg Met
      290      295      300
Ala Arg Ile Leu Gly Lys Leu Gly Ser Ile Asp Ala Asn Gln Gly Asp
305      310      315      320
Leu Leu Leu Gly Trp Asp Thr Asp Gln Phe Pro Thr Asn Ile Tyr Asp
      325      330      335
Thr Thr Leu Ala Met Tyr Glu Val Ile Lys Ala Gly Gly Phe Thr Lys
      340      345      350
Gly Gly Leu Asn Phe Asp Ala Lys Val Arg Arg Ala Ser Tyr Lys Val
      355      360      365
Glu Asp Leu Phe Ile Gly His Ile Ala Gly Met Asp Thr Phe Ala Leu
      370      375      380
Gly Phe Lys Ile Ala Tyr Lys Leu Ala Lys Asp Gly Val Phe Asp Lys
385      390      395      400
Phe Ile Glu Glu Lys Tyr Arg Ser Phe Lys Glu Gly Ile Gly Lys Glu
      405      410      415
Ile Val Glu Gly Lys Thr Asp Phe Glu Lys Leu Glu Glu Tyr Ile Ile
      420      425      430
Asp Lys Glu Asp Ile Glu Leu Pro Ser Gly Lys Gln Glu Tyr Leu Glu
      435      440      445
Ser Leu Leu Asn Ser Tyr Ile Val Lys Thr Ile Ala Glu Leu Arg Ser
      450      455      460
Glu Lys Asp Glu Leu
465

```

&lt;210&gt; 29

&lt;211&gt; 469

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 29

Met Arg Val Leu Leu Val Ala Leu Ala Leu Leu Ala Leu Ala Ala Ser

1		5		10		15									
Ala	Thr	Ser	Met	Ala	Glu	Phe	Phe	Pro	Glu	Ile	Pro	Lys	Val	Gln	Phe
			20					25					30		
Glu	Gly	Lys	Glu	Ser	Thr	Asn	Pro	Leu	Ala	Phe	Lys	Phe	Tyr	Asp	Pro
		35					40					45			
Glu	Glu	Ile	Ile	Asp	Gly	Lys	Pro	Leu	Lys	Asp	His	Leu	Lys	Phe	Ser
	50					55					60				
Val	Ala	Phe	Trp	His	Thr	Phe	Val	Asn	Glu	Gly	Arg	Asp	Pro	Phe	Gly
65					70					75					80
Asp	Pro	Thr	Ala	Asp	Arg	Pro	Trp	Asn	Arg	Tyr	Thr	Asp	Pro	Met	Asp
				85				90						95	
Lys	Ala	Phe	Ala	Arg	Val	Asp	Ala	Leu	Phe	Glu	Phe	Cys	Glu	Lys	Leu
			100					105					110		
Asn	Ile	Glu	Tyr	Phe	Cys	Phe	His	Asp	Arg	Asp	Ile	Ala	Pro	Glu	Gly
		115					120					125			
Lys	Thr	Leu	Arg	Glu	Thr	Asn	Lys	Ile	Leu	Asp	Lys	Val	Val	Glu	Arg
	130					135					140				
Ile	Lys	Glu	Arg	Met	Lys	Asp	Ser	Asn	Val	Lys	Leu	Leu	Trp	Gly	Thr
145					150					155					160
Ala	Asn	Leu	Phe	Ser	His	Pro	Arg	Tyr	Met	His	Gly	Ala	Ala	Thr	Thr
				165				170						175	
Cys	Ser	Ala	Asp	Val	Phe	Ala	Tyr	Ala	Ala	Ala	Gln	Val	Lys	Lys	Ala
			180					185					190		
Leu	Glu	Ile	Thr	Lys	Glu	Leu	Gly	Gly	Glu	Gly	Tyr	Val	Phe	Trp	Gly
	195						200					205			
Gly	Arg	Glu	Gly	Tyr	Glu	Thr	Leu	Leu	Asn	Thr	Asp	Leu	Gly	Phe	Glu
	210					215					220				
Leu	Glu	Asn	Leu	Ala	Arg	Phe	Leu	Arg	Met	Ala	Val	Asp	Tyr	Ala	Lys
225					230					235					240
Arg	Ile	Gly	Phe	Thr	Gly	Gln	Phe	Leu	Ile	Glu	Pro	Lys	Pro	Lys	Glu
				245				250						255	
Pro	Thr	Lys	His	Gln	Tyr	Asp	Phe	Asp	Val	Ala	Thr	Ala	Tyr	Ala	Phe
			260					265					270		
Leu	Lys	Ser	His	Gly	Leu	Asp	Glu	Tyr	Phe	Lys	Phe	Asn	Ile	Glu	Ala
	275						280					285			
Asn	His	Ala	Thr	Leu	Ala	Gly	His	Thr	Phe	Gln	His	Glu	Leu	Arg	Met
	290					295					300				
Ala	Arg	Ile	Leu	Gly	Lys	Leu	Gly	Ser	Ile	Asp	Ala	Asn	Gln	Gly	Asp
305					310					315					320
Leu	Leu	Leu	Gly	Trp	Asp	Thr	Asp	Gln	Phe	Pro	Thr	Asn	Val	Tyr	Asp
			325					330						335	
Thr	Thr	Leu	Ala	Met	Tyr	Glu	Val	Ile	Lys	Ala	Gly	Gly	Phe	Thr	Lys
			340					345					350		
Gly	Gly	Leu	Asn	Phe	Asp	Ala	Lys	Val	Arg	Arg	Ala	Ser	Tyr	Lys	Val
		355					360					365			
Glu	Asp	Leu	Phe	Ile	Gly	His	Ile	Ala	Gly	Met	Asp	Thr	Phe	Ala	Leu
	370					375					380				
Gly	Phe	Lys	Val	Ala	Tyr	Lys	Leu	Val	Lys	Asp	Gly	Val	Leu	Asp	Lys
385					390					395					400
Phe	Ile	Glu	Glu	Lys	Tyr	Arg	Ser	Phe	Arg	Glu	Gly	Ile	Gly	Arg	Asp
				405				410						415	
Ile	Val	Glu	Gly	Lys	Val	Asp	Phe	Glu	Lys	Leu	Glu	Glu	Tyr	Ile	Ile
		420						425					430		
Asp	Lys	Glu	Thr	Ile	Glu	Leu	Pro	Ser	Gly	Lys	Gln	Glu	Tyr	Leu	Glu



	435		440		445
Ser	Leu	Ile	Asn	Ser	Tyr
	450		455		460
Glu	Lys	Asp	Glu	Leu	
465					

<210> 30  
 <211> 463  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 30																	
Met	Arg	Val	Leu	Leu	Val	Ala	Leu	Ala	Leu	Leu	Ala	Leu	Ala	Ala	Ser		
1			5				10				15						
Ala	Thr	Ser	Met	Ala	Glu	Phe	Phe	Pro	Glu	Ile	Pro	Lys	Val	Gln	Phe		
		20					25				30						
Glu	Gly	Lys	Glu	Ser	Thr	Asn	Pro	Leu	Ala	Phe	Lys	Phe	Tyr	Asp	Pro		
		35				40					45						
Glu	Glu	Ile	Ile	Asp	Gly	Lys	Pro	Leu	Lys	Asp	His	Leu	Lys	Phe	Ser		
	50				55		60										
Val	Ala	Phe	Trp	His	Thr	Phe	Val	Asn	Glu	Gly	Arg	Asp	Pro	Phe	Gly		
65			70				75								80		
Asp	Pro	Thr	Ala	Asp	Arg	Pro	Trp	Asn	Arg	Tyr	Thr	Asp	Pro	Met	Asp		
		85				90						95					
Lys	Ala	Phe	Ala	Arg	Val	Asp	Ala	Leu	Phe	Glu	Phe	Cys	Glu	Lys	Leu		
		100				105						110					
Asn	Ile	Glu	Tyr	Phe	Cys	Phe	His	Asp	Arg	Asp	Ile	Ala	Pro	Glu	Gly		
		115				120					125						
Lys	Thr	Leu	Arg	Glu	Thr	Asn	Lys	Ile	Leu	Asp	Lys	Val	Val	Glu	Arg		
	130				135		140										
Ile	Lys	Glu	Arg	Met	Lys	Asp	Ser	Asn	Val	Lys	Leu	Leu	Trp	Gly	Thr		
145			150				155								160		
Ala	Asn	Leu	Phe	Ser	His	Pro	Arg	Tyr	Met	His	Gly	Ala	Ala	Thr	Thr		
		165				170								175			
Cys	Ser	Ala	Asp	Val	Phe	Ala	Tyr	Ala	Ala	Ala	Gln	Val	Lys	Lys	Ala		
		180				185					190						
Leu	Glu	Ile	Thr	Lys	Glu	Leu	Gly	Gly	Glu	Gly	Tyr	Val	Phe	Trp	Gly		
		195				200					205						
Gly	Arg	Glu	Gly	Tyr	Glu	Thr	Leu	Leu	Asn	Thr	Asp	Leu	Gly	Phe	Glu		
	210				215					220							
Leu	Glu	Asn	Leu	Ala	Arg	Phe	Leu	Arg	Met	Ala	Val	Asp	Tyr	Ala	Lys		
225			230				235								240		
Arg	Ile	Gly	Phe	Thr	Gly	Gln	Phe	Leu	Ile	Glu	Pro	Lys	Pro	Lys	Glu		
		245				250								255			
Pro	Thr	Lys	His	Gln	Tyr	Asp	Phe	Asp	Val	Ala	Thr	Ala	Tyr	Ala	Phe		
		260				265							270				
Leu	Lys	Ser	His	Gly	Leu	Asp	Glu	Tyr	Phe	Lys	Phe	Asn	Ile	Glu	Ala		
	275				280		285										
Asn	His	Ala	Thr	Leu	Ala	Gly	His	Thr	Phe	Gln	His	Glu	Leu	Arg	Met		
	290				295		300										

Ala Arg Ile Leu Gly Lys Leu Gly Ser Ile Asp Ala Asn Gln Gly Asp  
 305 310 315 320  
 Leu Leu Leu Gly Trp Asp Thr Asp Gln Phe Pro Thr Asn Val Tyr Asp  
 325 330 335  
 Thr Thr Leu Ala Met Tyr Glu Val Ile Lys Ala Gly Gly Phe Thr Lys  
 340 345 350  
 Gly Gly Leu Asn Phe Asp Ala Lys Val Arg Arg Ala Ser Tyr Lys Val  
 355 360 365  
 Glu Asp Leu Phe Ile Gly His Ile Ala Gly Met Asp Thr Phe Ala Leu  
 370 375 380  
 Gly Phe Lys Val Ala Tyr Lys Leu Val Lys Asp Gly Val Leu Asp Lys  
 385 390 395 400  
 Phe Ile Glu Glu Lys Tyr Arg Ser Phe Arg Glu Gly Ile Gly Arg Asp  
 405 410 415  
 Ile Val Glu Gly Lys Val Asp Phe Glu Lys Leu Glu Glu Tyr Ile Ile  
 420 425 430  
 Asp Lys Glu Thr Ile Glu Leu Pro Ser Gly Lys Gln Glu Tyr Leu Glu  
 435 440 445  
 Ser Leu Ile Asn Ser Tyr Ile Val Lys Thr Ile Leu Glu Leu Arg  
 450 455 460

<210> 31  
 <211> 25  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 31  
 Met Gly Lys Asn Gly Asn Leu Cys Cys Phe Ser Leu Leu Leu Leu Leu  
 1 5 10 15  
 Leu Ala Gly Leu Ala Ser Gly His Gln  
 20 25

<210> 32  
 <211> 30  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 32  
 Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu Leu Val Ser  
 1 5 10 15  
 Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg Ala  
 20 25 30

<210> 33  
 <211> 460

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 33

Met	Arg	Val	Leu	Leu	Val	Ala	Leu	Ala	Leu	Leu	Ala	Leu	Ala	Ala	Ser
1				5					10					15	
Ala	Thr	Ser	Ala	Lys	Tyr	Leu	Glu	Leu	Glu	Glu	Gly	Gly	Val	Ile	Met
			20					25					30		
Gln	Ala	Phe	Tyr	Trp	Asp	Val	Pro	Ser	Gly	Gly	Ile	Trp	Trp	Asp	Thr
		35					40					45			
Ile	Arg	Gln	Lys	Ile	Pro	Glu	Trp	Tyr	Asp	Ala	Gly	Ile	Ser	Ala	Ile
	50					55					60				
Trp	Ile	Pro	Pro	Ala	Ser	Lys	Gly	Met	Ser	Gly	Gly	Tyr	Ser	Met	Gly
65					70					75				80	
Tyr	Asp	Pro	Tyr	Asp	Tyr	Phe	Asp	Leu	Gly	Glu	Tyr	Tyr	Gln	Lys	Gly
				85					90					95	
Thr	Val	Glu	Thr	Arg	Phe	Gly	Ser	Lys	Gln	Glu	Leu	Ile	Asn	Met	Ile
			100					105						110	
Asn	Thr	Ala	His	Ala	Tyr	Gly	Ile	Lys	Val	Ile	Ala	Asp	Ile	Val	Ile
		115					120					125			
Asn	His	Arg	Ala	Gly	Gly	Asp	Leu	Glu	Trp	Asn	Pro	Phe	Val	Gly	Asp
	130					135					140				
Tyr	Thr	Trp	Thr	Asp	Phe	Ser	Lys	Val	Ala	Ser	Gly	Lys	Tyr	Thr	Ala
145					150					155					160
Asn	Tyr	Leu	Asp	Phe	His	Pro	Asn	Glu	Leu	His	Ala	Gly	Asp	Ser	Gly
				165				170						175	
Thr	Phe	Gly	Gly	Tyr	Pro	Asp	Ile	Cys	His	Asp	Lys	Ser	Trp	Asp	Gln
			180					185						190	
Tyr	Trp	Leu	Trp	Ala	Ser	Gln	Glu	Ser	Tyr	Ala	Ala	Tyr	Leu	Arg	Ser
		195					200					205			
Ile	Gly	Ile	Asp	Ala	Trp	Arg	Phe	Asp	Tyr	Val	Lys	Gly	Tyr	Gly	Ala
	210					215					220				
Trp	Val	Val	Lys	Asp	Trp	Leu	Asn	Trp	Trp	Gly	Gly	Trp	Ala	Val	Gly
225					230					235					240
Glu	Tyr	Trp	Asp	Thr	Asn	Val	Asp	Ala	Leu	Leu	Asn	Trp	Ala	Tyr	Ser
				245					250					255	
Ser	Gly	Ala	Lys	Val	Phe	Asp	Phe	Pro	Leu	Tyr	Tyr	Lys	Met	Asp	Ala
			260					265					270		
Ala	Phe	Asp	Asn	Lys	Asn	Ile	Pro	Ala	Leu	Val	Glu	Ala	Leu	Lys	Asn
		275					280					285			
Gly	Gly	Thr	Val	Val	Ser	Arg	Asp	Pro	Phe	Lys	Ala	Val	Thr	Phe	Val
	290					295					300				
Ala	Asn	His	Asp	Thr	Asp	Ile	Ile	Trp	Asn	Lys	Tyr	Pro	Ala	Tyr	Ala
305					310					315					320
Phe	Ile	Leu	Thr	Tyr	Glu	Gly	Gln	Pro	Thr	Ile	Phe	Tyr	Arg	Asp	Tyr
				325					330					335	
Glu	Glu	Trp	Leu	Asn	Lys	Asp	Lys	Leu	Lys	Asn	Leu	Ile	Trp	Ile	His
			340					345					350		
Asp	Asn	Leu	Ala	Gly	Gly	Ser	Thr	Ser	Ile	Val	Tyr	Tyr	Asp	Ser	Asp
		355					360					365			
Glu	Met	Ile	Phe	Val	Arg	Asn	Gly	Tyr	Gly	Ser	Lys	Pro	Gly	Leu	Ile

370	375	380
Thr Tyr Ile Asn Leu Gly Ser Ser Lys Val Gly Arg Trp Val Tyr Val		
385	390	395
Pro Lys Phe Ala Gly Ala Cys Ile His Glu Tyr Thr Gly Asn Leu Gly		400
	405	410
Gly Trp Val Asp Lys Tyr Val Tyr Ser Ser Gly Trp Val Tyr Leu Glu		415
	420	425
Ala Pro Ala Tyr Asp Pro Ala Asn Gly Gln Tyr Gly Tyr Ser Val Trp		430
	435	440
Ser Tyr Cys Gly Val Gly Ser Glu Lys Asp Glu Leu		445
450	455	460

<210> 34  
 <211> 825  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 34

Met Arg Val Leu Leu Val Ala Leu Ala Leu Leu Ala Leu Ala Ala Ser	
1	5
Ala Thr Ser Ala Gly His Trp Tyr Lys His Gln Arg Ala Tyr Gln Phe	10
	15
	20
Thr Gly Glu Asp Asp Phe Gly Lys Val Ala Val Val Lys Leu Pro Met	25
	30
	35
Asp Leu Thr Lys Val Gly Ile Ile Val Arg Leu Asn Glu Trp Gln Ala	40
	45
	50
Lys Asp Val Ala Lys Asp Arg Phe Ile Glu Ile Lys Asp Gly Lys Ala	55
	60
	65
Glu Val Trp Ile Leu Gln Gly Val Glu Glu Ile Phe Tyr Glu Lys Pro	70
	75
	80
	85
Asp Thr Ser Pro Arg Ile Phe Phe Ala Gln Ala Arg Ser Asn Lys Val	90
	95
	100
Ile Glu Ala Phe Leu Thr Asn Pro Val Asp Thr Lys Lys Lys Glu Leu	105
	110
	115
Phe Lys Val Thr Val Asp Gly Lys Glu Ile Pro Val Ser Arg Val Glu	120
	125
	130
Lys Ala Asp Pro Thr Asp Ile Asp Val Thr Asn Tyr Val Arg Ile Val	135
	140
	145
Leu Ser Glu Ser Leu Lys Glu Glu Asp Leu Arg Lys Asp Val Glu Leu	150
	155
	160
	165
Ile Ile Glu Gly Tyr Lys Pro Ala Arg Val Ile Met Met Glu Ile Leu	170
	175
	180
Asp Asp Tyr Tyr Tyr Asp Gly Glu Leu Gly Ala Val Tyr Ser Pro Glu	185
	190
	195
Lys Thr Ile Phe Arg Val Trp Ser Pro Val Ser Lys Trp Val Lys Val	200
	205
	210
Leu Leu Phe Lys Asn Gly Glu Asp Thr Glu Pro Tyr Gln Val Val Asn	215
	220
	225
Met Glu Tyr Lys Gly Asn Gly Val Trp Glu Ala Val Val Glu Gly Asp	230
	235
	240
	245
	250
	255

Leu	Asp	Gly	Val	Phe	Tyr	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Gly	Lys	Ile
			260					265					270		
Arg	Thr	Thr	Val	Asp	Pro	Tyr	Ser	Lys	Ala	Val	Tyr	Ala	Asn	Asn	Gln
		275					280					285			
Glu	Ser	Ala	Val	Val	Asn	Leu	Ala	Arg	Thr	Asn	Pro	Glu	Gly	Trp	Glu
	290					295					300				
Asn	Asp	Arg	Gly	Pro	Lys	Ile	Glu	Gly	Tyr	Glu	Asp	Ala	Ile	Ile	Tyr
305					310					315					320
Glu	Ile	His	Ile	Ala	Asp	Ile	Thr	Gly	Leu	Glu	Asn	Ser	Gly	Val	Lys
				325					330					335	
Asn	Lys	Gly	Leu	Tyr	Leu	Gly	Leu	Thr	Glu	Glu	Asn	Thr	Lys	Ala	Pro
			340					345					350		
Gly	Gly	Val	Thr	Thr	Gly	Leu	Ser	His	Leu	Val	Glu	Leu	Gly	Val	Thr
		355					360						365		
His	Val	His	Ile	Leu	Pro	Phe	Phe	Asp	Phe	Tyr	Thr	Gly	Asp	Glu	Leu
	370					375						380			
Asp	Lys	Asp	Phe	Glu	Lys	Tyr	Tyr	Asn	Trp	Gly	Tyr	Asp	Pro	Tyr	Leu
385					390					395					400
Phe	Met	Val	Pro	Glu	Gly	Arg	Tyr	Ser	Thr	Asp	Pro	Lys	Asn	Pro	His
			405						410					415	
Thr	Arg	Ile	Arg	Glu	Val	Lys	Glu	Met	Val	Lys	Ala	Leu	His	Lys	His
			420					425					430		
Gly	Ile	Gly	Val	Ile	Met	Asp	Met	Val	Phe	Pro	His	Thr	Tyr	Gly	Ile
		435					440						445		
Gly	Glu	Leu	Ser	Ala	Phe	Asp	Gln	Thr	Val	Pro	Tyr	Tyr	Phe	Tyr	Arg
	450					455					460				
Ile	Asp	Lys	Thr	Gly	Ala	Tyr	Leu	Asn	Glu	Ser	Gly	Cys	Gly	Asn	Val
465					470					475					480
Ile	Ala	Ser	Glu	Arg	Pro	Met	Met	Arg	Lys	Phe	Ile	Val	Asp	Thr	Val
			485						490					495	
Thr	Tyr	Trp	Val	Lys	Glu	Tyr	His	Ile	Asp	Gly	Phe	Arg	Phe	Asp	Gln
			500					505					510		
Met	Gly	Leu	Ile	Asp	Lys	Lys	Thr	Met	Leu	Glu	Val	Glu	Arg	Ala	Leu
		515					520					525			
His	Lys	Ile	Asp	Pro	Thr	Ile	Ile	Leu	Tyr	Gly	Glu	Pro	Trp	Gly	Gly
	530					535					540				
Trp	Gly	Ala	Pro	Ile	Arg	Phe	Gly	Lys	Ser	Asp	Val	Ala	Gly	Thr	His
545					550					555					560
Val	Ala	Ala	Phe	Asn	Asp	Glu	Phe	Arg	Asp	Ala	Ile	Arg	Gly	Ser	Val
			565						570					575	
Phe	Asn	Pro	Ser	Val	Lys	Gly	Phe	Val	Met	Gly	Gly	Tyr	Gly	Lys	Glu
			580					585					590		
Thr	Lys	Ile	Lys	Arg	Gly	Val	Val	Gly	Ser	Ile	Asn	Tyr	Asp	Gly	Lys
		595					600					605			
Leu	Ile	Lys	Ser	Phe	Ala	Leu	Asp	Pro	Glu	Glu	Thr	Ile	Asn	Tyr	Ala
	610					615					620				
Ala	Cys	His	Asp	Asn	His	Thr	Leu	Trp	Asp	Lys	Asn	Tyr	Leu	Ala	Ala
625					630					635					640
Lys	Ala	Asp	Lys	Lys	Lys	Glu	Trp	Thr	Glu	Glu	Glu	Leu	Lys	Asn	Ala
			645						650					655	
Gln	Lys	Leu	Ala	Gly	Ala	Ile	Leu	Leu	Thr	Ser	Gln	Gly	Val	Pro	Phe
			660				665						670		
Leu	His	Gly	Gly	Gln	Asp	Phe	Cys	Arg	Thr	Thr	Asn	Phe	Asn	Asp	Asn
		675					680					685			

Ser	Tyr	Asn	Ala	Pro	Ile	Ser	Ile	Asn	Gly	Phe	Asp	Tyr	Glu	Arg	Lys
690						695					700				
Leu	Gln	Phe	Ile	Asp	Val	Phe	Asn	Tyr	His	Lys	Gly	Leu	Ile	Lys	Leu
705					710					715					720
Arg	Lys	Glu	His	Pro	Ala	Phe	Arg	Leu	Lys	Asn	Ala	Glu	Glu	Ile	Lys
				725						730					735
Lys	His	Leu	Glu	Phe	Leu	Pro	Gly	Gly	Arg	Arg	Ile	Val	Ala	Phe	Met
			740					745					750		
Leu	Lys	Asp	His	Ala	Gly	Gly	Asp	Pro	Trp	Lys	Asp	Ile	Val	Val	Ile
		755					760					765			
Tyr	Asn	Gly	Asn	Leu	Glu	Lys	Thr	Thr	Tyr	Lys	Leu	Pro	Glu	Gly	Lys
	770					775						780			
Trp	Asn	Val	Val	Val	Asn	Ser	Gln	Lys	Ala	Gly	Thr	Glu	Val	Ile	Glu
785					790					795					800
Thr	Val	Glu	Gly	Thr	Ile	Glu	Leu	Asp	Pro	Leu	Ser	Ala	Tyr	Val	Leu
				805					810					815	
Tyr	Arg	Glu	Ser	Glu	Lys	Asp	Glu	Leu							
			820					825							

<210> 35  
 <211> 460  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 35															
Met	Arg	Val	Leu	Leu	Val	Ala	Leu	Ala	Leu	Leu	Ala	Leu	Ala	Ala	Ser
1				5				10						15	
Ala	Thr	Ser	Ala	Lys	Tyr	Leu	Glu	Leu	Glu	Glu	Gly	Gly	Val	Ile	Met
			20					25					30		
Gln	Ala	Phe	Tyr	Trp	Asp	Val	Pro	Ser	Gly	Gly	Ile	Trp	Trp	Asp	Thr
		35				40					45				
Ile	Arg	Gln	Lys	Ile	Pro	Glu	Trp	Tyr	Asp	Ala	Gly	Ile	Ser	Ala	Ile
	50					55					60				
Trp	Ile	Pro	Pro	Ala	Ser	Lys	Gly	Met	Ser	Gly	Gly	Tyr	Ser	Met	Gly
65					70					75					80
Tyr	Asp	Pro	Tyr	Asp	Tyr	Phe	Asp	Leu	Gly	Glu	Tyr	Tyr	Gln	Lys	Gly
			85						90					95	
Thr	Val	Glu	Thr	Arg	Phe	Gly	Ser	Lys	Gln	Glu	Leu	Ile	Asn	Met	Ile
			100					105						110	
Asn	Thr	Ala	His	Ala	Tyr	Gly	Ile	Lys	Val	Ile	Ala	Asp	Ile	Val	Ile
		115					120						125		
Asn	His	Arg	Ala	Gly	Gly	Asp	Leu	Glu	Trp	Asn	Pro	Phe	Val	Gly	Asp
	130					135					140				
Tyr	Thr	Trp	Thr	Asp	Phe	Ser	Lys	Val	Ala	Ser	Gly	Lys	Tyr	Thr	Ala
145					150					155					160
Asn	Tyr	Leu	Asp	Phe	His	Pro	Asn	Glu	Leu	His	Ala	Gly	Asp	Ser	Gly
			165						170					175	
Thr	Phe	Gly	Gly	Tyr	Pro	Asp	Ile	Cys	His	Asp	Lys	Ser	Trp	Asp	Gln
		180						185					190		
Tyr	Trp	Leu	Trp	Ala	Ser	Gln	Glu	Ser	Tyr	Ala	Ala	Tyr	Leu	Arg	Ser



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Glu His Ile Ile Gly Leu Gly Glu Lys Ala Phe Glu Leu Asp Arg Lys  
                                   85                                  90                                  95  
 Arg Lys Arg Tyr Val Met Tyr Asn Val Asp Ala Gly Ala Tyr Lys Lys  
                                   100                                  105                                  110  
 Tyr Gln Asp Pro Leu Tyr Val Ser Ile Pro Leu Phe Ile Ser Val Lys  
                                   115                                  120                                  125  
 Asp Gly Val Ala Thr Gly Tyr Phe Phe Asn Ser Ala Ser Lys Val Ile  
                                   130                                  135                                  140  
 Phe Asp Val Gly Leu Glu Glu Tyr Asp Lys Val Ile Val Thr Ile Pro  
 145                                  150                                  155                                  160  
 Glu Asp Ser Val Glu Phe Tyr Val Ile Glu Gly Pro Arg Ile Glu Asp  
                                   165                                  170                                  175  
 Val Leu Glu Lys Tyr Thr Glu Leu Thr Gly Lys Pro Phe Leu Pro Pro  
                                   180                                  185                                  190  
 Met Trp Ala Phe Gly Tyr Met Ile Ser Arg Tyr Ser Tyr Tyr Pro Gln  
                                   195                                  200                                  205  
 Asp Lys Val Val Glu Leu Val Asp Ile Met Gln Lys Glu Gly Phe Arg  
                                   210                                  215                                  220  
 Val Ala Gly Val Phe Leu Asp Ile His Tyr Met Asp Ser Tyr Lys Leu  
 225                                  230                                  235                                  240  
 Phe Thr Trp His Pro Tyr Arg Phe Pro Glu Pro Lys Lys Leu Ile Asp  
                                   245                                  250                                  255  
 Glu Leu His Lys Arg Asn Val Lys Leu Ile Thr Ile Val Asp His Gly  
                                   260                                  265                                  270  
 Ile Arg Val Asp Gln Asn Tyr Ser Pro Phe Leu Ser Gly Met Gly Lys  
                                   275                                  280                                  285  
 Phe Cys Glu Ile Glu Ser Gly Glu Leu Phe Val Gly Lys Met Trp Pro  
                                   290                                  295                                  300  
 Gly Thr Thr Val Tyr Pro Asp Phe Phe Arg Glu Asp Thr Arg Glu Trp  
 305                                  310                                  315                                  320  
 Trp Ala Gly Leu Ile Ser Glu Trp Leu Ser Gln Gly Val Asp Gly Ile  
                                   325                                  330                                  335  
 Trp Leu Asp Met Asn Glu Pro Thr Asp Phe Ser Arg Ala Ile Glu Ile  
                                   340                                  345                                  350  
 Arg Asp Val Leu Ser Ser Leu Pro Val Gln Phe Arg Asp Asp Arg Leu  
                                   355                                  360                                  365  
 Val Thr Thr Phe Pro Asp Asn Val Val His Tyr Leu Arg Gly Lys Arg  
                                   370                                  375                                  380  
 Val Lys His Glu Lys Val Arg Asn Ala Tyr Pro Leu Tyr Glu Ala Met  
 385                                  390                                  395                                  400  
 Ala Thr Phe Lys Gly Phe Arg Thr Ser His Arg Asn Glu Ile Phe Ile  
                                   405                                  410                                  415  
 Leu Ser Arg Ala Gly Tyr Ala Gly Ile Gln Arg Tyr Ala Phe Ile Trp  
                                   420                                  425                                  430  
 Thr Gly Asp Asn Thr Pro Ser Trp Asp Asp Leu Lys Leu Gln Leu Gln  
                                   435                                  440                                  445  
 Leu Val Leu Gly Leu Ser Ile Ser Gly Val Pro Phe Val Gly Cys Asp  
                                   450                                  455                                  460  
 Ile Gly Gly Phe Gln Gly Arg Asn Phe Ala Glu Ile Asp Asn Ser Met  
 465                                  470                                  475                                  480  
 Asp Leu Leu Val Lys Tyr Tyr Ala Leu Ala Leu Phe Phe Pro Phe Tyr  
                                   485                                  490                                  495  
 Arg Ser His Lys Ala Thr Asp Gly Ile Asp Thr Glu Pro Val Phe Leu  
                                   500                                  505                                  510

Pro Asp Tyr Tyr Lys Glu Lys Val Lys Glu Ile Val Glu Leu Arg Tyr  
 515 520 525  
 Lys Phe Leu Pro Tyr Ile Tyr Ser Leu Ala Leu Glu Ala Ser Glu Lys  
 530 535 540  
 Gly His Pro Val Ile Arg Pro Leu Phe Tyr Glu Phe Gln Asp Asp Asp  
 545 550 555 560  
 Asp Met Tyr Arg Ile Glu Asp Glu Tyr Met Val Gly Lys Tyr Leu Leu  
 565 570 575  
 Tyr Ala Pro Ile Val Ser Lys Glu Glu Ser Arg Leu Val Thr Leu Pro  
 580 585 590  
 Arg Gly Lys Trp Tyr Asn Tyr Trp Asn Gly Glu Ile Ile Asn Gly Lys  
 595 600 605  
 Ser Val Val Lys Ser Thr His Glu Leu Pro Ile Tyr Leu Arg Glu Gly  
 610 615 620  
 Ser Ile Ile Pro Leu Glu Gly Asp Glu Leu Ile Val Tyr Gly Glu Thr  
 625 630 635 640  
 Ser Phe Lys Arg Tyr Asp Asn Ala Glu Ile Thr Ser Ser Ser Asn Glu  
 645 650 655  
 Ile Lys Phe Ser Arg Glu Ile Tyr Val Ser Lys Leu Thr Ile Thr Ser  
 660 665 670  
 Glu Lys Pro Val Ser Lys Ile Ile Val Asp Asp Ser Lys Glu Ile Gln  
 675 680 685  
 Val Glu Lys Thr Met Gln Asn Thr Tyr Val Ala Lys Ile Asn Gln Lys  
 690 695 700  
 Ile Arg Gly Lys Ile Asn Leu Glu Ser Glu Lys Asp Glu Leu  
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&lt;210&gt; 37

&lt;211&gt; 1434

&lt;212&gt; DNA

<213> *Thermotoga maritima*

&lt;400&gt; 37

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 accctggtgc cacgcggttc catggccgag ttcttcccgg agatcccga gatccagttc 120  
 gagggcaagg agtccaccaa cccgctcgcc ttccgcttct acgaccgaa cgagggtgatc 180  
 gacggcaagc cgctcaagga ccacctcaag ttctccgtgg ccttctggca caccttcgtg 240  
 aacgagggcc gcgaccggtt cggcgacccg accgcccagc gcccggtggaa ccgcttctcc 300  
 gacccgatgg acaaggcctt cgcccgcgtg gacgcccctt tgcagttctg cgagaagctc 360  
 aacatcgagt acttctgctt ccacgaccgc gacatcgccc cggaggggcaa gacctccgc 420  
 gagaccaaca agatcctcga caaggtgggtg gagcgcacatc aggagcgcat gaaggactcc 480  
 aacgtgaagc tcctctgggg caccgccaac ctcttctccc acccgcgcta catgcacggc 540  
 gccgccacca cctgctccgc cgacgtgttc gcctacgccc ccgcccaggt gaagaaggcc 600  
 ctggagatca ccaaggagct gggcggcgag ggctacgtgt tctggggcgg ccgagaggcc 660  
 tacgagaccc tcctcaacac cgacctcggc ctggagctgg agaacctcgc ccgcttcctc 720  
 cgcatggccg tggagtacgc caagaagatc ggcttcaccg gccagttcct catcgagccg 780  
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 ctgcgccggc acaccttcca gcacgagctg cgcatggccc gcatcctcgg caagctcggc 960  
 tccatcgacg ccaaccaggg cgacctcctc ctccggtggg acaccgacca gttcccgacc 1020  
 aacatctacg acaccaccct cgccatgtac gaggtgatca aggccggcgg cttcaccaag 1080  
 ggccggcctc acttcgacgc caaggtgcgc cgccctcctt acaaggtgga ggacctcttc 1140  
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<210> 38
<211> 477
<212> PRT
<213> Thermotoga maritima
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210

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		355						360					365				
Val	Arg	Arg	Ala	Ser	Tyr	Lys	Val	Glu	Asp	Leu	Phe	Ile	Gly	His	Ile		
		370						375					380				
Ala	Gly	Met	Asp	Thr	Phe	Ala	Leu	Gly	Phe	Lys	Ile	Ala	Tyr	Lys	Leu		
385						390				395					400		
Ala	Lys	Asp	Gly	Val	Phe	Asp	Lys	Phe	Ile	Glu	Glu	Lys	Tyr	Arg	Ser		
			405					410						415			
Phe	Lys	Glu	Gly	Ile	Gly	Lys	Glu	Ile	Val	Glu	Gly	Lys	Thr	Asp	Phe		
		420						425					430				
Glu	Lys	Leu	Glu	Glu	Tyr	Ile	Ile	Asp	Lys	Glu	Asp	Ile	Glu	Leu	Pro		
		435					440					445					
Ser	Gly	Lys	Gln	Glu	Tyr	Leu	Glu	Ser	Leu	Leu	Asn	Ser	Tyr	Ile	Val		
	450					455					460						
Lys	Thr	Ile	Ala	Glu	Leu	Arg	Ser	Glu	Lys	Asp	Glu	Leu					
465					470					475							

&lt;210&gt; 39

&lt;211&gt; 1434

&lt;212&gt; DNA

&lt;213&gt; Thermotoga neapolitana

&lt;400&gt; 39

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gagggcaagg agtccaccaa cccgctcgcc ttcaagttct acgaccgga ggagatcatc 180
gacggcaagc cgctcaagga ccacctcaag ttctccgtgg ccttctggca caccttcgtg 240
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gacccgatgg acaaggcctt cgcccgcgtg gacgcccctc tcgagttctg cgagaagctc 360
aacatcgagt acttctgctt ccacgaccgc gacatcgccc cggagggcaa gaccctccgc 420
gagaccaaca agatcctcga caagggtggtg gagcgcatca aggagcgcat gaaggactcc 480
aacgtgaagc tcctctgggg caccgccaac ctcttctccc acccgcgcta catgcacggc 540
gccgccacca cctgctccgc cgacgtgttc gcctacgccc ccgccaggt gaagaaggcc 600
ctggagatca ccaaggagct gggcggcgag ggctacgtgt tctggggcgg ccgcgagggc 660
tacgagaccc tcctcaacac cgacctcggc ttcgagctgg agaacctcgc ccgcttctc 720
cgcatggccg tggactacgc caagcgcata ggcttcaccg gccagttcct catcgagccg 780
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&lt;210&gt; 40

&lt;211&gt; 477

&lt;212&gt; PRT

&lt;213&gt; Thermotoga neapolitana

&lt;400&gt; 40

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Pro	Asp	Leu	Gly	Thr	Leu	Val	Pro	Arg	Gly	Ser	Met	Ala	Glu	Phe	Phe
			20					25					30		
Pro	Glu	Ile	Pro	Lys	Val	Gln	Phe	Glu	Gly	Lys	Glu	Ser	Thr	Asn	Pro
		35					40					45			
Leu	Ala	Phe	Lys	Phe	Tyr	Asp	Pro	Glu	Glu	Ile	Ile	Asp	Gly	Lys	Pro
	50					55					60				
Leu	Lys	Asp	His	Leu	Lys	Phe	Ser	Val	Ala	Phe	Trp	His	Thr	Phe	Val
65					70					75					80
Asn	Glu	Gly	Arg	Asp	Pro	Phe	Gly	Asp	Pro	Thr	Ala	Asp	Arg	Pro	Trp
				85					90					95	
Asn	Arg	Tyr	Thr	Asp	Pro	Met	Asp	Lys	Ala	Phe	Ala	Arg	Val	Asp	Ala
			100					105					110		
Leu	Phe	Glu	Phe	Cys	Glu	Lys	Leu	Asn	Ile	Glu	Tyr	Phe	Cys	Phe	His
		115					120					125			
Asp	Arg	Asp	Ile	Ala	Pro	Glu	Gly	Lys	Thr	Leu	Arg	Glu	Thr	Asn	Lys
	130					135						140			
Ile	Leu	Asp	Lys	Val	Val	Glu	Arg	Ile	Lys	Glu	Arg	Met	Lys	Asp	Ser
145					150					155					160
Asn	Val	Lys	Leu	Leu	Trp	Gly	Thr	Ala	Asn	Leu	Phe	Ser	His	Pro	Arg
				165					170					175	
Tyr	Met	His	Gly	Ala	Ala	Thr	Thr	Cys	Ser	Ala	Asp	Val	Phe	Ala	Tyr
			180					185						190	
Ala	Ala	Ala	Gln	Val	Lys	Lys	Ala	Leu	Glu	Ile	Thr	Lys	Glu	Leu	Gly
		195					200					205			
Gly	Glu	Gly	Tyr	Val	Phe	Trp	Gly	Gly	Arg	Glu	Gly	Tyr	Glu	Thr	Leu
	210					215					220				
Leu	Asn	Thr	Asp	Leu	Gly	Phe	Glu	Leu	Glu	Asn	Leu	Ala	Arg	Phe	Leu
225					230					235					240
Arg	Met	Ala	Val	Asp	Tyr	Ala	Lys	Arg	Ile	Gly	Phe	Thr	Gly	Gln	Phe
				245					250					255	
Leu	Ile	Glu	Pro	Lys	Pro	Lys	Glu	Pro	Thr	Lys	His	Gln	Tyr	Asp	Phe
			260					265					270		
Asp	Val	Ala	Thr	Ala	Tyr	Ala	Phe	Leu	Lys	Ser	His	Gly	Leu	Asp	Glu
		275					280					285			
Tyr	Phe	Lys	Phe	Asn	Ile	Glu	Ala	Asn	His	Ala	Thr	Leu	Ala	Gly	His
	290					295					300				
Thr	Phe	Gln	His	Glu	Leu	Arg	Met	Ala	Arg	Ile	Leu	Gly	Lys	Leu	Gly
305					310					315					320
Ser	Ile	Asp	Ala	Asn	Gln	Gly	Asp	Leu	Leu	Leu	Gly	Trp	Asp	Thr	Asp
				325					330					335	
Gln	Phe	Pro	Thr	Asn	Val	Tyr	Asp	Thr	Thr	Leu	Ala	Met	Tyr	Glu	Val
			340					345					350		
Ile	Lys	Ala	Gly	Gly	Phe	Thr	Lys	Gly	Gly	Leu	Asn	Phe	Asp	Ala	Lys
		355					360					365			
Val	Arg	Arg	Ala	Ser	Tyr	Lys	Val	Glu	Asp	Leu	Phe	Ile	Gly	His	Ile
	370					375					380				
Ala	Gly	Met	Asp	Thr	Phe	Ala	Leu	Gly	Phe	Lys	Val	Ala	Tyr	Lys	Leu
385					390					395					400
Val	Lys	Asp	Gly	Val	Leu	Asp	Lys	Phe	Ile	Glu	Glu	Lys	Tyr	Arg	Ser
				405					410					415	



Phe Arg Glu Gly Ile Gly Arg Asp Ile Val Glu Gly Lys Val Asp Phe  
                   420                  425                  430  
 Glu Lys Leu Glu Glu Tyr Ile Ile Asp Lys Glu Thr Ile Glu Leu Pro  
                   435                  440                  445  
 Ser Gly Lys Gln Glu Tyr Leu Glu Ser Leu Ile Asn Ser Tyr Ile Val  
                   450                  455                  460  
 Lys Thr Ile Leu Glu Leu Arg Ser Glu Lys Asp Glu Leu  
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<210> 41  
 <211> 1435  
 <212> DNA  
 <213> Thermotoga maritima

<400> 41  
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 gagatcccga agatccagtt cgagggcaag gagtccacca acccgctcgc cttccgcttc 180  
 tacgacccga acgaggtgat cgacggcaag ccgctcaagg accacctcaa gttctccgtg 240  
 gccttctggc acaccttcgt gaacgagggc cgcgaccgt tggcgaccc gaccgcccag 300  
 cgcccgtgga accgcttctc cgacccgatg gacaaggcct tcgcccgcgt ggacgcccctc 360  
 ttcgagttct gcgagaagct caacatcgag tacttctgct tccacgaccg cgacatcccc 420  
 cggaggggcaa gacctccgc gagaccaaca agatcctcga caaggtgggtg gagcgcatca 480  
 aggagcgcac gaaggactcc aacgtgaagc tcctctgggg caccgccaac ctcttctccc 540  
 acccgcgcta catgcacggc gccgccacca cctgctccgc cgacgtgttc gcctacgccg 600  
 ccgcccaggt gaagaaggcc ctggagatca ccaaggagct gggcgggcgag ggctacgtgt 660  
 tctggggcgg ccgcgagggc tacgagaccc tcctcaacac cgacctcggc ctggagctgg 720  
 agaacctcgc ccgcttcctc cgcattggccg tggagtacgc caagaagatc ggcttcaccg 780  
 gccagttcct catcgagccg aagccgaagg agccgaccaa gcaccagtac gcttcgacgt 840  
 ggccaccgcc tacgccttcc tcaagaacca cggcctcgac gagtacttca agttcaacat 900  
 cgaggccaac cagccacccc tcgcccggca cacttccag cagagctgc gcatggccc 960  
 catcctcggc aagctcggct ccacgcagc caaccagggc gacctcctc tcggctggga 1020  
 caccgaccag ttcccgaaca acatctacga caccacctc gccatgtacg aggtgatcaa 1080  
 ggccggcggc ttcaccaagg gcggcctcaa cttcgacgcc aaggtgcgcc gcgcctccta 1140  
 caaggtggag gacctcttca tcggccacat cgccggcatg gacaccttcg ccctcggctt 1200  
 caagatcgcc tacaagctcg ccaaggacgg cgtgttcgac aagttcatcg aggagaagta 1260  
 ccgctccttc aaggagggca tcggcaagga gatcgtggag ggcaagaccg acttcgagaa 1320  
 gctggaggag tacatcatcg acaaggagga catcgagctg ccgtccggca agcaggagta 1380  
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<210> 42  
 <211> 478  
 <212> PRT  
 <213> Thermotoga maritima

<400> 42  
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                   20                  25                  30  
 Ile Pro Met Ala Glu Phe Phe Pro Glu Ile Pro Lys Ile Gln Phe Glu  
                   35                  40                  45  
 Gly Lys Glu Ser Thr Asn Pro Leu Ala Phe Arg Phe Tyr Asp Pro Asn

50	55	60															
Glu	Val	Ile	Asp	Gly	Lys	Pro	Leu	Lys	Asp	His	Leu	Lys	Phe	Ser	Val		
65					70					75					80		
Ala	Phe	Trp	His	Thr	Phe	Val	Asn	Glu	Gly	Arg	Asp	Pro	Phe	Gly	Asp		
				85					90					95			
Pro	Thr	Ala	Glu	Arg	Pro	Trp	Asn	Arg	Phe	Ser	Asp	Pro	Met	Asp	Lys		
			100					105					110				
Ala	Phe	Ala	Arg	Val	Asp	Ala	Leu	Phe	Glu	Phe	Cys	Glu	Lys	Leu	Asn		
		115					120					125					
Ile	Glu	Tyr	Phe	Cys	Phe	His	Asp	Arg	Asp	Ile	Ala	Pro	Glu	Gly	Lys		
	130					135					140						
Thr	Leu	Arg	Glu	Thr	Asn	Lys	Ile	Leu	Asp	Lys	Val	Val	Glu	Arg	Ile		
145					150					155					160		
Lys	Glu	Arg	Met	Lys	Asp	Ser	Asn	Val	Lys	Leu	Leu	Trp	Gly	Thr	Ala		
				165					170					175			
Asn	Leu	Phe	Ser	His	Pro	Arg	Tyr	Met	His	Gly	Ala	Ala	Thr	Thr	Cys		
			180					185					190				
Ser	Ala	Asp	Val	Phe	Ala	Tyr	Ala	Ala	Ala	Gln	Val	Lys	Lys	Ala	Leu		
		195					200					205					
Glu	Ile	Thr	Lys	Glu	Leu	Gly	Gly	Glu	Gly	Tyr	Val	Phe	Trp	Gly	Gly		
	210					215					220						
Arg	Glu	Gly	Tyr	Glu	Thr	Leu	Leu	Asn	Thr	Asp	Leu	Gly	Leu	Glu	Leu		
225					230					235					240		
Glu	Asn	Leu	Ala	Arg	Phe	Leu	Arg	Met	Ala	Val	Glu	Tyr	Ala	Lys	Lys		
				245					250					255			
Ile	Gly	Phe	Thr	Gly	Gln	Phe	Leu	Ile	Glu	Pro	Lys	Pro	Lys	Glu	Pro		
			260					265					270				
Thr	Lys	His	Gln	Tyr	Asp	Phe	Asp	Val	Ala	Thr	Ala	Tyr	Ala	Phe	Leu		
		275					280					285					
Lys	Asn	His	Gly	Leu	Asp	Glu	Tyr	Phe	Lys	Phe	Asn	Ile	Glu	Ala	Asn		
	290					295					300						
His	Ala	Thr	Leu	Ala	Gly	His	Thr	Phe	Gln	His	Glu	Leu	Arg	Met	Ala		
305					310					315					320		
Arg	Ile	Leu	Gly	Lys	Leu	Gly	Ser	Ile	Asp	Ala	Asn	Gln	Gly	Asp	Leu		
				325					330					335			
Leu	Leu	Gly	Trp	Asp	Thr	Asp	Gln	Phe	Pro	Thr	Asn	Ile	Tyr	Asp	Thr		
			340					345					350				
Thr	Leu	Ala	Met	Tyr	Glu	Val	Ile	Lys	Ala	Gly	Gly	Phe	Thr	Lys	Gly		
	355						360					365					
Gly	Leu	Asn	Phe	Asp	Ala	Lys	Val	Arg	Arg	Ala	Ser	Tyr	Lys	Val	Glu		
	370					375					380						
Asp	Leu	Phe	Ile	Gly	His	Ile	Ala	Gly	Met	Asp	Thr	Phe	Ala	Leu	Gly		
385					390					395					400		
Phe	Lys	Ile	Ala	Tyr	Lys	Leu	Ala	Lys	Asp	Gly	Val	Phe	Asp	Lys	Phe		
				405					410					415			
Ile	Glu	Glu	Lys	Tyr	Arg	Ser	Phe	Lys	Glu	Gly	Ile	Gly	Lys	Glu	Ile		
			420					425					430				
Val	Glu	Gly	Lys	Thr	Asp	Phe	Glu	Lys	Leu	Glu	Glu	Tyr	Ile	Ile	Asp		
	435						440					445					
Lys	Glu	Asp	Ile	Glu	Leu	Pro	Ser	Gly	Lys	Gln	Glu	Tyr	Leu	Glu	Ser		
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Leu	Leu	Asn	Ser	Tyr	Ile	Val	Lys	Thr	Ile	Ala	Glu	Leu	Arg				
465					470				475								

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 <211> 1436  
 <212> DNA  
 <213> Thermotoga neapolitana

<400> 43  
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 gagatcccga aggtgcagtt cgagggcaag gagtccacca acccgctcgc cttcaagtcc 180  
 tacgaccccg aggagatcat cgacggcaag ccgctcaagg accacctcaa gttctccgtg 240  
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 cgcccggtga accgctacac cgacccgatg gacaaggcct tcgcccgcgt ggacgcccctc 360  
 ttcgagttct gcgagaagct caacatcgag tacttctgct tccacgaccg cgacatcccc 420  
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 aggagcgcac gaaggactcc aacgtgaagc tcctctgggg caccgccaac ctcttctccc 540  
 acccgcgcta catgcacggc gccgccacca cctgctccgc cgacgtgttc gcctacgccg 600  
 ccgcccaggt gaagaaggcc ctggagatca ccaaggagct gggcggcgag ggctacgtgt 660  
 tctggggcgg ccgagagggc tacgagaccc tcctcaacac cgacctcggc ttcgagctgg 720  
 agaacctcgc ccgcttcctc cgcattggccg tggactacgc caagcgcacg ggcttcaccg 780  
 gccagttcct catcgagccg aagccgaagg agccgaccaa gcaccagtac gacttcgacg 840  
 tggccaccgc ctacgccttc ctcaagtccc acggcctcga cgagtacttc aagttcaaca 900  
 tcgaggccaa ccacgccacc ctcgccggcc acacctcca gcacgagctg cgcattggcc 960  
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 acaccgacca gttcccgaac aacgtgtacg acaccacct cgccatgtac gaggtgatca 1080  
 aggccggcgg cttcaccaag ggcggcctca acttcgacgc caaggtgcgc cgcgcctcct 1140  
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 agctggagga gtacatcatc gacaaggaga ccatcgagct gccgtccggc aagcaggagt 1380  
 acctggagtc cctcatcaac tcctacatcg tgaagaccat cctggagctg cgctga 1436

<210> 44  
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 <212> PRT  
 <213> Thermotoga neapolitana

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 Ile Pro Met Ala Glu Phe Phe Pro Glu Ile Pro Lys Val Gln Phe Glu  
 35 40 45  
 Gly Lys Glu Ser Thr Asn Pro Leu Ala Phe Lys Phe Tyr Asp Pro Glu  
 50 55 60  
 Glu Ile Ile Asp Gly Lys Pro Leu Lys Asp His Leu Lys Phe Ser Val  
 65 70 75 80  
 Ala Phe Trp His Thr Phe Val Asn Glu Gly Arg Asp Pro Phe Gly Asp  
 85 90 95  
 Pro Thr Ala Asp Arg Pro Trp Asn Arg Tyr Thr Asp Pro Met Asp Lys  
 100 105 110  
 Ala Phe Ala Arg Val Asp Ala Leu Phe Glu Phe Cys Glu Lys Leu Asn  
 115 120 125

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Ile Glu Tyr Phe Cys Phe His Asp Arg Asp Ile Ala Pro Glu Gly Lys
 130          135          140
Thr Leu Arg Glu Thr Asn Lys Ile Leu Asp Lys Val Val Glu Arg Ile
145          150          155          160
Lys Glu Arg Met Lys Asp Ser Asn Val Lys Leu Leu Trp Gly Thr Ala
          165          170          175
Asn Leu Phe Ser His Pro Arg Tyr Met His Gly Ala Ala Thr Thr Cys
          180          185          190
Ser Ala Asp Val Phe Ala Tyr Ala Ala Ala Gln Val Lys Lys Ala Leu
          195          200          205
Glu Ile Thr Lys Glu Leu Gly Gly Glu Gly Tyr Val Phe Trp Gly Gly
 210          215          220
Arg Glu Gly Tyr Glu Thr Leu Leu Asn Thr Asp Leu Gly Phe Glu Leu
225          230          235          240
Glu Asn Leu Ala Arg Phe Leu Arg Met Ala Val Asp Tyr Ala Lys Arg
          245          250          255
Ile Gly Phe Thr Gly Gln Phe Leu Ile Glu Pro Lys Pro Lys Glu Pro
          260          265          270
Thr Lys His Gln Tyr Asp Phe Asp Val Ala Thr Ala Tyr Ala Phe Leu
          275          280          285
Lys Ser His Gly Leu Asp Glu Tyr Phe Lys Phe Asn Ile Glu Ala Asn
 290          295          300
His Ala Thr Leu Ala Gly His Thr Phe Gln His Glu Leu Arg Met Ala
305          310          315          320
Arg Ile Leu Gly Lys Leu Gly Ser Ile Asp Ala Asn Gln Gly Asp Leu
          325          330          335
Leu Leu Gly Trp Asp Thr Asp Gln Phe Pro Thr Asn Val Tyr Asp Thr
 340          345          350
Thr Leu Ala Met Tyr Glu Val Ile Lys Ala Gly Gly Phe Thr Lys Gly
 355          360          365
Gly Leu Asn Phe Asp Ala Lys Val Arg Arg Ala Ser Tyr Lys Val Glu
 370          375          380
Asp Leu Phe Ile Gly His Ile Ala Gly Met Asp Thr Phe Ala Leu Gly
385          390          395          400
Phe Lys Val Ala Tyr Lys Leu Val Lys Asp Gly Val Leu Asp Lys Phe
          405          410          415
Ile Glu Glu Lys Tyr Arg Ser Phe Arg Glu Gly Ile Gly Arg Asp Ile
 420          425          430
Val Glu Gly Lys Val Asp Phe Glu Lys Leu Glu Glu Tyr Ile Ile Asp
 435          440          445
Lys Glu Thr Ile Glu Leu Pro Ser Gly Lys Gln Glu Tyr Leu Glu Ser
 450          455          460
Leu Ile Asn Ser Tyr Ile Val Lys Thr Ile Leu Glu Leu Arg
465          470          475

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<210> 45  
 <211> 1095  
 <212> PRT  
 <213> Aspergillus shirousami

<400> 45  
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			20					25					30		
Ala	Asp	Gln	Lys	Tyr	Cys	Gly	Gly	Thr	Trp	Gln	Gly	Ile	Ile	Asp	Lys
		35				40					45				
Leu	Asp	Tyr	Ile	Gln	Gly	Met	Gly	Phe	Thr	Ala	Ile	Trp	Ile	Thr	Pro
	50				55					60					
Val	Thr	Ala	Gln	Leu	Pro	Gln	Thr	Thr	Ala	Tyr	Gly	Asp	Ala	Tyr	His
65				70						75					80
Gly	Tyr	Trp	Gln	Gln	Asp	Ile	Tyr	Ser	Leu	Asn	Glu	Asn	Tyr	Gly	Thr
			85					90					95		
Ala	Asp	Asp	Leu	Lys	Ala	Leu	Ser	Ser	Ala	Leu	His	Glu	Arg	Gly	Met
			100				105						110		
Tyr	Leu	Met	Val	Asp	Val	Val	Ala	Asn	His	Met	Gly	Tyr	Asp	Gly	Ala
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Gly	Ser	Ser	Val	Asp	Tyr	Ser	Val	Phe	Lys	Pro	Phe	Ser	Ser	Gln	Asp
	130					135					140				
Tyr	Phe	His	Pro	Phe	Cys	Phe	Ile	Gln	Asn	Tyr	Glu	Asp	Gln	Thr	Gln
145					150					155					160
Val	Glu	Asp	Cys	Trp	Leu	Gly	Asp	Asn	Thr	Val	Ser	Leu	Pro	Asp	Leu
			165					170						175	
Asp	Thr	Thr	Lys	Asp	Val	Val	Lys	Asn	Glu	Trp	Tyr	Asp	Trp	Val	Gly
			180					185					190		
Ser	Leu	Val	Ser	Asn	Tyr	Ser	Ile	Asp	Gly	Leu	Arg	Ile	Asp	Thr	Val
		195					200					205			
Lys	His	Val	Gln	Lys	Asp	Phe	Trp	Pro	Gly	Tyr	Asn	Lys	Ala	Ala	Gly
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Val	Tyr	Cys	Ile	Gly	Glu	Val	Leu	Asp	Val	Asp	Pro	Ala	Tyr	Thr	Cys
225					230					235					240
Pro	Tyr	Gln	Asn	Val	Met	Asp	Gly	Val	Leu	Asn	Tyr	Pro	Ile	Tyr	Tyr
			245					250						255	
Pro	Leu	Leu	Asn	Ala	Phe	Lys	Ser	Thr	Ser	Gly	Ser	Met	Asp	Asp	Leu
			260					265					270		
Tyr	Asn	Met	Ile	Asn	Thr	Val	Lys	Ser	Asp	Cys	Pro	Asp	Ser	Thr	Leu
		275					280					285			
Leu	Gly	Thr	Phe	Val	Glu	Asn	His	Asp	Asn	Pro	Arg	Phe	Ala	Ser	Tyr
	290					295				300					
Thr	Asn	Asp	Ile	Ala	Leu	Ala	Lys	Asn	Val	Ala	Ala	Phe	Ile	Ile	Leu
305					310					315					320
Asn	Asp	Gly	Ile	Pro	Ile	Ile	Tyr	Ala	Gly	Gln	Glu	Gln	His	Tyr	Ala
			325						330					335	
Gly	Gly	Asn	Asp	Pro	Ala	Asn	Arg	Glu	Ala	Thr	Trp	Leu	Ser	Gly	Tyr
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Pro	Thr	Asp	Ser	Glu	Leu	Tyr	Lys	Leu	Ile	Ala	Ser	Ala	Asn	Ala	Ile
		355					360					365			
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Trp	Pro	Ile	Tyr	Lys	Asp	Asp	Thr	Thr	Ile	Ala	Met	Arg	Lys	Gly	Thr
385					390					395					400
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Asp	Ser	Tyr	Thr	Leu	Ser	Leu	Ser	Gly	Ala	Gly	Tyr	Thr	Ala	Gly	Gln
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Gln	Leu	Thr	Glu	Val	Ile	Gly	Cys	Thr	Thr	Val	Thr	Val	Gly	Ser	Asp
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Gly	Asn	Val	Pro	Val	Pro	Met	Ala	Gly	Gly	Leu	Pro	Arg	Val	Leu	Tyr
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Ala	Thr	Leu	Asp	Ser	Trp	Leu	Ser	Asn	Glu	Ala	Thr	Val	Ala	Arg	Thr
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Ala	Ile	Leu	Asn	Asn	Ile	Gly	Ala	Asp	Gly	Ala	Trp	Val	Ser	Gly	Ala
			500					505					510		
Asp	Ser	Gly	Ile	Val	Val	Ala	Ser	Pro	Ser	Thr	Asp	Asn	Pro	Asp	Tyr
		515					520					525			
Phe	Tyr	Thr	Trp	Thr	Arg	Asp	Ser	Gly	Ile	Val	Leu	Lys	Thr	Leu	Val
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Asp	Leu	Phe	Arg	Asn	Gly	Asp	Thr	Asp	Leu	Leu	Ser	Thr	Ile	Glu	His
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Tyr	Ile	Ser	Ser	Gln	Ala	Ile	Ile	Gln	Gly	Val	Ser	Asn	Pro	Ser	Gly
				565					570					575	
Asp	Leu	Ser	Ser	Gly	Gly	Leu	Gly	Glu	Pro	Lys	Phe	Asn	Val	Asp	Glu
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Thr	Ala	Tyr	Ala	Gly	Ser	Trp	Gly	Arg	Pro	Gln	Arg	Asp	Gly	Pro	Ala
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Trp	Glu	Glu	Val	Asn	Gly	Ser	Ser	Phe	Phe	Thr	Ile	Ala	Val	Gln	His
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Ser	Phe	Trp	Thr	Gly	Ser	Tyr	Ile	Leu	Ala	Asn	Phe	Asp	Ser	Ser	Arg
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Ser	Gly	Lys	Asp	Thr	Asn	Thr	Leu	Leu	Gly	Ser	Ile	His	Thr	Phe	Asp
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Ala	Leu	Ala	Asn	His	Lys	Glu	Val	Val	Asp	Ser	Phe	Arg	Ser	Ile	Tyr
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Thr	Leu	Asn	Asp	Gly	Leu	Ser	Asp	Ser	Glu	Ala	Val	Ala	Val	Gly	Arg
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Tyr	Pro	Glu	Asp	Ser	Tyr	Tyr	Asn	Gly	Asn	Pro	Trp	Phe	Leu	Cys	Thr
785					790					795					800
Leu	Ala	Ala	Ala	Glu	Gln	Leu	Tyr	Asp	Ala	Leu	Tyr	Gln	Trp	Asp	Lys
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Gln	Gly	Ser	Leu	Glu	Ile	Thr	Asp	Val	Ser	Leu	Asp	Phe	Phe	Lys	Ala
			820					825					830		
Leu	Tyr	Ser	Gly	Ala	Ala	Thr	Gly	Thr	Tyr	Ser	Ser	Ser	Ser	Ser	Thr
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Tyr	Ser	Ser	Ile	Val	Ser	Ala	Val	Lys	Thr	Phe	Ala	Asp	Gly	Phe	Val
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Ser	Ile	Val	Glu	Thr	His	Ala	Ala	Ser	Asn	Gly	Ser	Leu	Ser	Glu	Gln
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219

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&lt;210&gt; 47

&lt;211&gt; 679

&lt;212&gt; PRT

&lt;213&gt; Thermoanaerobacterium thermosaccharolyticum

&lt;400&gt; 47

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Val Leu Ser Gly Cys Ser Asn Asn Val Ser Ser Ile Lys Ile Asp Arg
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Phe Asn Asn Ile Ser Ala Val Asn Gly Pro Gly Glu Glu Asp Thr Trp
          20          25          30
Ala Ser Ala Gln Lys Gln Gly Val Gly Thr Ala Asn Asn Tyr Val Ser
          35          40          45
Arg Val Trp Phe Thr Leu Ala Asn Gly Ala Ile Ser Glu Val Tyr Tyr
          50          55          60
Pro Thr Ile Asp Thr Ala Asp Val Lys Glu Ile Lys Phe Ile Val Thr
65          70          75          80

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Asp	Gly	Lys	Ser	Phe	Val	Ser	Asp	Glu	Thr	Lys	Asp	Ala	Ile	Ser	Lys	85	90	95
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Asp	Lys	Lys	Gly	Arg	Tyr	Arg	Ile	Thr	Lys	Glu	Ile	Phe	Thr	Asp	Val	115	120	125
Lys	Arg	Asn	Ser	Leu	Ile	Met	Lys	Ala	Lys	Phe	Glu	Ala	Leu	Glu	Gly	130	135	140
Ser	Ile	His	Asp	Tyr	Lys	Leu	Tyr	Leu	Ala	Tyr	Asp	Pro	His	Ile	Lys	145	150	155
Asn	Gln	Gly	Ser	Tyr	Asn	Glu	Gly	Tyr	Val	Ile	Lys	Ala	Asn	Asn	Asn	165	170	175
Glu	Met	Leu	Met	Ala	Lys	Arg	Asp	Asn	Val	Tyr	Thr	Ala	Leu	Ser	Ser	180	185	190
Asn	Ile	Gly	Trp	Lys	Gly	Tyr	Ser	Ile	Gly	Tyr	Tyr	Lys	Val	Asn	Asp	195	200	205
Ile	Met	Thr	Asp	Leu	Asp	Glu	Asn	Lys	Gln	Met	Thr	Lys	His	Tyr	Asp	210	215	220
Ser	Ala	Arg	Gly	Asn	Ile	Ile	Glu	Gly	Ala	Glu	Ile	Asp	Leu	Thr	Lys	225	230	235
Asn	Ser	Glu	Phe	Glu	Ile	Val	Leu	Ser	Phe	Gly	Gly	Ser	Asp	Ser	Glu	245	250	255
Ala	Ala	Lys	Thr	Ala	Leu	Glu	Thr	Leu	Gly	Glu	Asp	Tyr	Asn	Asn	Leu	260	265	270
Lys	Asn	Asn	Tyr	Ile	Asp	Glu	Trp	Thr	Lys	Tyr	Cys	Asn	Thr	Leu	Asn	275	280	285
Asn	Phe	Asn	Gly	Lys	Ala	Asn	Ser	Leu	Tyr	Tyr	Asn	Ser	Met	Met	Ile	290	295	300
Leu	Lys	Ala	Ser	Glu	Asp	Lys	Thr	Asn	Lys	Gly	Ala	Tyr	Ile	Ala	Ser	305	310	315
Leu	Ser	Ile	Pro	Trp	Gly	Asp	Gly	Gln	Arg	Asp	Asp	Asn	Thr	Gly	Gly	325	330	335
Tyr	His	Leu	Val	Trp	Ser	Arg	Asp	Leu	Tyr	His	Val	Ala	Asn	Ala	Phe	340	345	350
Ile	Ala	Ala	Gly	Asp	Val	Asp	Ser	Ala	Asn	Arg	Ser	Leu	Asp	Tyr	Leu	355	360	365
Ala	Lys	Val	Val	Lys	Asp	Asn	Gly	Met	Ile	Pro	Gln	Asn	Thr	Trp	Ile	370	375	380
Ser	Gly	Lys	Pro	Tyr	Trp	Thr	Ser	Ile	Gln	Leu	Asp	Glu	Gln	Ala	Asp	385	390	395
Pro	Ile	Ile	Leu	Ser	Tyr	Arg	Leu	Lys	Arg	Tyr	Asp	Leu	Tyr	Asp	Ser	405	410	415
Leu	Val	Lys	Pro	Leu	Ala	Asp	Phe	Ile	Ile	Lys	Ile	Gly	Pro	Lys	Thr	420	425	430
Gly	Gln	Glu	Arg	Trp	Glu	Glu	Ile	Gly	Gly	Tyr	Ser	Pro	Ala	Thr	Met	435	440	445
Ala	Ala	Glu	Val	Ala	Gly	Leu	Thr	Cys	Ala	Ala	Tyr	Ile	Ala	Glu	Gln	450	455	460
Asn	Lys	Asp	Tyr	Glu	Ser	Ala	Gln	Lys	Tyr	Gln	Glu	Lys	Ala	Asp	Asn	465	470	475
Trp	Gln	Lys	Leu	Ile	Asp	Asn	Leu	Thr	Tyr	Thr	Glu	Asn	Gly	Pro	Leu	485	490	495
Gly	Asn	Gly	Gln	Tyr	Tyr	Ile	Arg	Ile	Ala	Gly	Leu	Ser	Asp	Pro	Asn	500	505	510

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Lys	Glu	Ile	Val	Asp	Pro	Ser	Phe	Leu	Glu	Leu	Val	Arg	Leu	Gly	Val
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Ser	Thr	Ile	Lys	Val	Asp	Thr	Pro	Lys	Gly	Pro	Ser	Trp	Tyr	Arg	Tyr
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Asn	His	Asp	Gly	Tyr	Gly	Glu	Pro	Ser	Lys	Thr	Glu	Leu	Tyr	His	Gly
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Ala	Gly	Lys	Gly	Arg	Leu	Trp	Pro	Leu	Leu	Thr	Gly	Glu	Arg	Gly	Met
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Tyr	Glu	Ile	Ala	Ala	Gly	Lys	Asp	Ala	Thr	Pro	Tyr	Val	Lys	Ala	Met
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Asp	Met	Pro	Asp	Ile	Val	Tyr									
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&lt;210&gt; 48

&lt;211&gt; 2037

&lt;212&gt; DNA

&lt;213&gt; Thermoanaerobacterium thermosaccharolyticum

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 48

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gtgaaggcga tggagaagtt cgccaacgag ggcggcatca tctccgagca ggtgtgggag 1920
gacaccggcc tcccgaaccga ctccgcctcc ccgtcaact gggcccacgc cgagtacgtg 1980
atcctcttcg cctccaacat cgagcacaag gtgctcgaca tgccggacat cgtgtac 2037

```

&lt;210&gt; 49

&lt;211&gt; 579

&lt;212&gt; PRT

<213> *Rhizopus oryzae*

&lt;400&gt; 49

```

Ala Ser Ile Pro Ser Ser Ala Ser Val Gln Leu Asp Ser Tyr Asn Tyr
1      5      10      15
Asp Gly Ser Thr Phe Ser Gly Lys Ile Tyr Val Lys Asn Ile Ala Tyr
20     25     30
Ser Lys Lys Val Thr Val Ile Tyr Ala Asp Gly Ser Asp Asn Trp Asn
35     40     45
Asn Asn Gly Asn Thr Ile Ala Ala Ser Tyr Ser Ala Pro Ile Ser Gly
50     55     60
Ser Asn Tyr Glu Tyr Trp Thr Phe Ser Ala Ser Ile Asn Gly Ile Lys
65     70     75     80
Glu Phe Tyr Ile Lys Tyr Glu Val Ser Gly Lys Thr Tyr Tyr Asp Asn
85     90     95
Asn Asn Ser Ala Asn Tyr Gln Val Ser Thr Ser Lys Pro Thr Thr Thr
100    105    110
Thr Ala Thr Ala Thr Thr Thr Thr Ala Pro Ser Thr Ser Thr Thr Thr
115    120    125
Pro Pro Ser Arg Ser Glu Pro Ala Thr Phe Pro Thr Gly Asn Ser Thr
130    135    140
Ile Ser Ser Trp Ile Lys Lys Gln Glu Gly Ile Ser Arg Phe Ala Met
145    150    155    160
Leu Arg Asn Ile Asn Pro Pro Gly Ser Ala Thr Gly Phe Ile Ala Ala
165    170    175
Ser Leu Ser Thr Ala Gly Pro Asp Tyr Tyr Tyr Ala Trp Thr Arg Asp
180    185    190
Ala Ala Leu Thr Ser Asn Val Ile Val Tyr Glu Tyr Asn Thr Thr Leu
195    200    205
Ser Gly Asn Lys Thr Ile Leu Asn Val Leu Lys Asp Tyr Val Thr Phe
210    215    220
Ser Val Lys Thr Gln Ser Thr Ser Thr Val Cys Asn Cys Leu Gly Glu
225    230    235    240
Pro Lys Phe Asn Pro Asp Ala Ser Gly Tyr Thr Gly Ala Trp Gly Arg
245    250    255
Pro Gln Asn Asp Gly Pro Ala Glu Arg Ala Thr Thr Phe Ile Leu Phe
260    265    270

```



Ala Asp Ser Tyr Leu Thr Gln Thr Lys Asp Ala Ser Tyr Val Thr Gly  
 275 280 285  
 Thr Leu Lys Pro Ala Ile Phe Lys Asp Leu Asp Tyr Val Val Asn Val  
 290 295 300  
 Trp Ser Asn Gly Cys Phe Asp Leu Trp Glu Glu Val Asn Gly Val His  
 305 310 315 320  
 Phe Tyr Thr Leu Met Val Met Arg Lys Gly Leu Leu Leu Gly Ala Asp  
 325 330 335  
 Phe Ala Lys Arg Asn Gly Asp Ser Thr Arg Ala Ser Thr Tyr Ser Ser  
 340 345 350  
 Thr Ala Ser Thr Ile Ala Asn Lys Ile Ser Ser Phe Trp Val Ser Ser  
 355 360 365  
 Asn Asn Trp Ile Gln Val Ser Gln Ser Val Thr Gly Gly Val Ser Lys  
 370 375 380  
 Lys Gly Leu Asp Val Ser Thr Leu Leu Ala Ala Asn Leu Gly Ser Val  
 385 390 395 400  
 Asp Asp Gly Phe Phe Thr Pro Gly Ser Glu Lys Ile Leu Ala Thr Ala  
 405 410 415  
 Val Ala Val Glu Asp Ser Phe Ala Ser Leu Tyr Pro Ile Asn Lys Asn  
 420 425 430  
 Leu Pro Ser Tyr Leu Gly Asn Ser Ile Gly Arg Tyr Pro Glu Asp Thr  
 435 440 445  
 Tyr Asn Gly Asn Gly Asn Ser Gln Gly Asn Ser Trp Phe Leu Ala Val  
 450 455 460  
 Thr Gly Tyr Ala Glu Leu Tyr Tyr Arg Ala Ile Lys Glu Trp Ile Gly  
 465 470 475 480  
 Asn Gly Gly Val Thr Val Ser Ser Ile Ser Leu Pro Phe Phe Lys Lys  
 485 490 495  
 Phe Asp Ser Ser Ala Thr Ser Gly Lys Lys Tyr Thr Val Gly Thr Ser  
 500 505 510  
 Asp Phe Asn Asn Leu Ala Gln Asn Ile Ala Leu Ala Ala Asp Arg Phe  
 515 520 525  
 Leu Ser Thr Val Gln Leu His Ala His Asn Asn Gly Ser Leu Ala Glu  
 530 535 540  
 Glu Phe Asp Arg Thr Thr Gly Leu Ser Thr Gly Ala Arg Asp Leu Thr  
 545 550 555 560  
 Trp Ser His Ala Ser Leu Ile Thr Ala Ser Tyr Ala Lys Ala Gly Ala  
 565 570 575  
 Pro Ala Ala

<210> 50  
 <211> 1737  
 <212> DNA  
 <213> *Rhizopus oryzae*

<400> 50  
 gcctccatcc cgctcctccgc ctccgtgcag ctcgactcct acaactacga cggctccacc 60  
 ttctccggca aaatctacgt gaagaacatc gcctactcca agaaggtgac cgtgatctac 120  
 gccgacggct ccgacaactg gaacaacaac ggcaacacca tcgccgcctc ctactccgcc 180  
 ccgatctccg gctccaacta cgagtactgg accttctccg cctccatcaa cggcatcaag 240  
 gagttctaca tcaagtacga ggtgtccggc aagacctact acgacaacaa caactccgcc 300  
 aactaccagg tgtccacctc caagccgacc accaccaccg ccaccgccac caccaccacc 360



```

gccccgtcca cctccaccac caccgccg ccccgtccg agccggccac cttcccgacc 420
ggcaactcca ccatctctc ctggatcaag aagcaggagg gcatctccc cttcgccatg 480
ctccgcaaca tcaaccgcc gggctccgcc accggcttca tcgccgctc cctctccacc 540
gccggcccgg actactacta cgcctggacc cgcgacgcg ccctcacctc caacgtgatc 600
gtgtacgagt acaacaccac cctctccggc aacaagacca tcctcaacgt gctcaaggac 660
tacgtgacct tctccgtgaa gacccagtcc acctccaccg tgtgcaactg cctcggcgag 720
ccgaagtcca acccggacgc ctccggctac accggcgctt gggggccgcc gcagaacgac 780
ggcccgccg agcgcgccac caccttcatc ctcttcgccg actcctacct caccagacc 840
aaggacgcct cctacgtgac cggcaccctc aagccggcca tcttcaagga cctcgactac 900
gtggtgaacg tgtggtccaa cggctgcttc gacctctggg aggaggtgaa cggcgtgcac 960
ttctacaccc tcatggtgat gcgcaagggc ctctctctcg gcgccgactt cgccaagcgc 1020
aacggcgact ccacccgcgc ctccacctac tcctccaccg cctccaccat cgccaacaaa 1080
atctctctct tctgggtgtc ctccaacaac tggatacagg tgtcccagtc cgtgaccggc 1140
ggcgtgtcca agaagggcct cgacgtgtcc acctctctcg ccgccaacct cggctccgtg 1200
gacgacggct tcttcacccc gggctccgag aagatcctcg ccaccgccgt ggccgtggag 1260
gactccttcg cctccctcta cccgatcaac aagaacctcc cgtcctacct cggcaactcc 1320
atcgcccgct acccggagga cacctacaac ggcaacggca actcccaggg caactcctgg 1380
ttctctcgcc tgaccggcta cgcgagctg tactaccgcg ccatcaagga gtggatcggc 1440
aacggcgggc tgaccgtgtc ctccatctcc ctcccgttct tcaagaagtt cgactcctcc 1500
gccacctccg gcaagaagta caccgtgggc acctccgact tcaacaacct cgcccagAAC 1560
atcgccctcg ccgccgaccg cttctctctc accgtgcagc tccacgcccc caacaacggc 1620
tccctcgccg aggagttcga ccgcaccacc ggctctcca ccggcgcccc cgacctcacc 1680
tggtcccacg cctccctcat caccgcctcc tacgccaagg ccggcgcccc ggccgcc 1737

```

<210> 51

<211> 439

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic

<400> 51

```

Met Ala Lys His Leu Ala Ala Met Cys Trp Cys Ser Leu Leu Val Leu
1          5          10          15
Val Leu Leu Cys Leu Gly Ser Gln Leu Ala Gln Ser Gln Val Leu Phe
20          25          30
Gln Gly Phe Asn Trp Glu Ser Trp Lys Lys Gln Gly Gly Trp Tyr Asn
35          40          45
Tyr Leu Leu Gly Arg Val Asp Asp Ile Ala Ala Thr Gly Ala Thr His
50          55          60
Val Trp Leu Pro Gln Pro Ser His Ser Val Ala Pro Gln Gly Tyr Met
65          70          75          80
Pro Gly Arg Leu Tyr Asp Leu Asp Ala Ser Lys Tyr Gly Thr His Ala
85          90          95
Glu Leu Lys Ser Leu Thr Ala Ala Phe His Ala Lys Gly Val Gln Cys
100         105         110
Val Ala Asp Val Val Ile Asn His Arg Cys Ala Asp Tyr Lys Asp Gly
115         120         125
Arg Gly Ile Tyr Cys Val Phe Glu Gly Gly Thr Pro Asp Ser Arg Leu
130         135         140
Asp Trp Gly Pro Asp Met Ile Cys Ser Asp Asp Thr Gln Tyr Ser Asn
145         150         155         160
Gly Arg Gly His Arg Asp Thr Gly Ala Asp Phe Ala Ala Ala Pro Asp

```

				165				170					175				
Ile	Asp	His	Leu	Asn	Pro	Arg	Val	Gln	Gln	Glu	Leu	Ser	Asp	Trp	Leu		
			180					185					190				
Asn	Trp	Leu	Lys	Ser	Asp	Leu	Gly	Phe	Asp	Gly	Trp	Arg	Leu	Asp	Phe		
		195					200					205					
Ala	Lys	Gly	Tyr	Ser	Ala	Ala	Val	Ala	Lys	Val	Tyr	Val	Asp	Ser	Thr		
	210					215					220						
Ala	Pro	Thr	Phe	Val	Val	Ala	Glu	Ile	Trp	Ser	Ser	Leu	His	Tyr	Asp		
225					230					235					240		
Gly	Asn	Gly	Glu	Pro	Ser	Ser	Asn	Gln	Asp	Ala	Asp	Arg	Gln	Glu	Leu		
				245				250					255				
Val	Asn	Trp	Ala	Gln	Ala	Val	Gly	Gly	Pro	Ala	Ala	Ala	Phe	Asp	Phe		
		260					265						270				
Thr	Thr	Lys	Gly	Val	Leu	Gln	Ala	Ala	Val	Gln	Gly	Glu	Leu	Trp	Arg		
		275					280					285					
Met	Lys	Asp	Gly	Asn	Gly	Lys	Ala	Pro	Gly	Met	Ile	Gly	Trp	Leu	Pro		
	290				295						300						
Glu	Lys	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Thr	Gly	Ser	Thr	Gln		
305					310					315					320		
Asn	Ser	Trp	Pro	Phe	Pro	Ser	Asp	Lys	Val	Met	Gln	Gly	Tyr	Ala	Tyr		
				325				330						335			
Ile	Leu	Thr	His	Pro	Gly	Thr	Pro	Cys	Ile	Phe	Tyr	Asp	His	Val	Phe		
		340					345						350				
Asp	Trp	Asn	Leu	Lys	Gln	Glu	Ile	Ser	Ala	Leu	Ser	Ala	Val	Arg	Ser		
	355						360					365					
Arg	Asn	Gly	Ile	His	Pro	Gly	Ser	Glu	Leu	Asn	Ile	Leu	Ala	Ala	Asp		
	370					375					380						
Gly	Asp	Leu	Tyr	Val	Ala	Lys	Ile	Asp	Asp	Lys	Val	Ile	Val	Lys	Ile		
385					390					395					400		
Gly	Ser	Arg	Tyr	Asp	Val	Gly	Asn	Leu	Ile	Pro	Ser	Asp	Phe	His	Ala		
				405				410					415				
Val	Ala	His	Gly	Asn	Asn	Tyr	Cys	Val	Trp	Glu	Lys	His	Gly	Leu	Arg		
		420					425						430				
Val	Pro	Ala	Gly	Arg	His	His											
		435															

&lt;210&gt; 52

&lt;211&gt; 1320

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

&lt;400&gt; 52

```

atggcgaagc acttggtgctgc catgtgctggtg tgcagcctcc tagtgcttgt actgctctgc 60
ttgggctccc agctggccca atcccagggtc ctcttccagg ggttcaactg ggagtcgtgg 120
aagaagcaag gtgggtggta caactacctc ctggggcggtg tggacgacat cgccgcgacg 180
ggggccacgc acgtctggct cccgcagccg tcgcactcgg tggcgccgca ggggtacatg 240
cccggccggc tctacgacct ggacgcgtcc aagtacggca cccacgcgga gctcaagtcg 300
ctcaccgcgg cgttccacgc caagggcggtc cagtgcgtcg cgcacgtcgt gatcaaccac 360
cgctgcgccg actacaagga cggccgcggc atctactgcg tcttcgaggg cggcacgccc 420
gacagccggc tcgactgggg ccccgcacatg atctgcagcg acgacacgca gtactccaac 480

```

```

gggcgcgggc accgcgacac gggggccgac ttgcgcgcgc cgcccgacat cgaccacctc 540
aaccgcgcgc tgcagcagga gctctcggac tggctcaact ggctcaagtc cgacctcggc 600
ttcgacggct ggcgcctcga cttcgccaag ggctactccg ccgccgtcgc caaggtgtac 660
gtcgacagca ccgccccac cttcgtcgtc gccgagatat ggagctccct ccactacgac 720
ggcaacggcg agccgtccag caaccaggac gccgacaggc aggagctggg caactgggcg 780
caggcgggtg gcggccccgc cgcggcggtc gacttcacca ccaagggcgt gctgcaggcg 840
gccgtccagg gcgagctgtg gcgcatgaag gacggcaacg gcaaggcgcc cgggatgatc 900
ggctggctgc cggagaaggc cgtcacgttc gtcgacaacc acgacaccgg ctccacgcag 960
aactcgtggc cattccccctc cgacaaggtc atgcagggtc acgcctatat cctcacgcac 1020
ccaggaaactc catgcatctt ctacgaccac gttttcgact ggaacctgaa gcaggagatc 1080
agcgcgctgt ctgcggtgag gtcaagaaac gggatccacc cggggagcga gctgaacatc 1140
ctcgccgcgc acggggatct ctacgtcgcc aagattgacg acaagggtcat cgtgaagatc 1200
gggtcacggc acgacgtcgg gaacctgatc ccctcagact tccacgccgt tgcccctggc 1260
aacaactact gcgtttggga gaagcacggc ctgagagttc cagcggggcg gcaccactag 1320

```

<210> 53  
 <211> 45  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic

<400> 53  
 Ala Thr Gly Gly Thr Thr Thr Thr Ala Thr Thr Thr Gly Ser Gly Gly  
 1 5 10 15  
 Val Thr Ser Thr Ser Lys Thr Thr Thr Thr Ala Ser Lys Thr Ser Thr  
 20 25 30  
 Thr Thr Ser Ser Thr Ser Cys Thr Thr Pro Thr Ala Val  
 35 40 45

<210> 54  
 <211> 137  
 <212> DNA  
 <213> Artificial Sequence  
 <220>  
 <223> synthetic

```

<400> 54
gccaccggcg gcaccaccac caccgccacc accaccggct ccggcggcgt gacctccacc 60
tccaagacca ccaccaccgc ctccaagacc tccaccacca cctcctccac ctctgcacc 120
accccgaccg ccgtgtc 137

```

<210> 55  
 <211> 300  
 <212> PRT  
 <213> Pyrococcus furiosus

<400> 55  
 Ile Tyr Phe Val Glu Lys Tyr His Thr Ser Glu Asp Lys Ser Thr Ser  
 1 5 10 15  
 Asn Thr Ser Ser Thr Pro Pro Gln Thr Thr Leu Ser Thr Thr Lys Val

			20					25					30				
Leu	Lys	Ile	Arg	Tyr	Pro	Asp	Asp	Gly	Glu	Trp	Pro	Gly	Ala	Pro	Ile		
		35					40					45					
Asp	Lys	Asp	Gly	Asp	Gly	Asn	Pro	Glu	Phe	Tyr	Ile	Glu	Ile	Asn	Leu		
	50					55					60						
Trp	Asn	Ile	Leu	Asn	Ala	Thr	Gly	Phe	Ala	Glu	Met	Thr	Tyr	Asn	Leu		
65				70						75					80		
Thr	Ser	Gly	Val	Leu	His	Tyr	Val	Gln	Gln	Leu	Asp	Asn	Ile	Val	Leu		
			85					90					95				
Arg	Asp	Arg	Ser	Asn	Trp	Val	His	Gly	Tyr	Pro	Glu	Ile	Phe	Tyr	Gly		
			100					105					110				
Asn	Lys	Pro	Trp	Asn	Ala	Asn	Tyr	Ala	Thr	Asp	Gly	Pro	Ile	Pro	Leu		
		115					120					125					
Pro	Ser	Lys	Val	Ser	Asn	Leu	Thr	Asp	Phe	Tyr	Leu	Thr	Ile	Ser	Tyr		
	130					135					140						
Lys	Leu	Glu	Pro	Lys	Asn	Gly	Leu	Pro	Ile	Asn	Phe	Ala	Ile	Glu	Ser		
145					150					155					160		
Trp	Leu	Thr	Arg	Glu	Ala	Trp	Arg	Thr	Thr	Gly	Ile	Asn	Ser	Asp	Glu		
			165					170						175			
Gln	Glu	Val	Met	Ile	Trp	Ile	Tyr	Tyr	Asp	Gly	Leu	Gln	Pro	Ala	Gly		
		180						185					190				
Ser	Lys	Val	Lys	Glu	Ile	Val	Val	Pro	Ile	Ile	Val	Asn	Gly	Thr	Pro		
	195					200						205					
Val	Asn	Ala	Thr	Phe	Glu	Val	Trp	Lys	Ala	Asn	Ile	Gly	Trp	Glu	Tyr		
	210					215					220						
Val	Ala	Phe	Arg	Ile	Lys	Thr	Pro	Ile	Lys	Glu	Gly	Thr	Val	Thr	Ile		
225					230					235					240		
Pro	Tyr	Gly	Ala	Phe	Ile	Ser	Val	Ala	Ala	Asn	Ile	Ser	Ser	Leu	Pro		
			245					250						255			
Asn	Tyr	Thr	Glu	Leu	Tyr	Leu	Glu	Asp	Val	Glu	Ile	Gly	Thr	Glu	Phe		
		260					265					270					
Gly	Thr	Pro	Ser	Thr	Thr	Ser	Ala	His	Leu	Glu	Trp	Trp	Ile	Thr	Asn		
		275				280						285					
Ile	Thr	Leu	Thr	Pro	Leu	Asp	Arg	Pro	Leu	Ile	Ser						
	290					295					300						

&lt;210&gt; 56

&lt;211&gt; 903

&lt;212&gt; DNA

<213> *Pyrococcus furiosus*

&lt;400&gt; 56

```

atctacttcg tggagaagta ccacacctcc gaggacaagt ccacctccaa cacctcctcc 60
accccgccgc agaccaccct ctccaccacc aaggtgctca agatccgcta cccggacgac 120
ggcgagtggc ccggcgcccc gatcgacaag gacggcgacg gcaaccgga gttctacatc 180
gagatcaacc tctggaacat cctcaacgcc accggcttcg ccgagatgac ctacaacctc 240
actagtggcg tgctccacta cgtgcagcag ctcgacaaca tcgtgctccg cgaccgctcc 300
aactgggtgc acggctaccc ggaaatcttc tacggcaaca agcgtggaa cgccaactac 360
gccaccgacg gccgatccc gctcccgtcc aaggtgtcca acctcaccga cttctacctc 420
accatctcct acaagctcga gccgaagaac ggtctcccga tcaacttcgc catcgagtcc 480
tggctcaccg gcgaggcctg gcgaccacc ggcataact ccgacgagca ggaggtgatg 540
atctggatct actacgacgg cctccagccc gcgggctcca aggtgaagga gatcgtgggtg 600
ccgatcatcg tgaacggcac cccggtgaac gccaccttcg aggtgtggaa ggccaacatc 660
ggctgggagt acgtggcctt ccgcatcaag accccgatca aggagggcac cgtgaccatc 720

```

```

ccgtacggcg ccttcatctc cgtggccgcc aacatctcct ccctcccgaa ctacaccgag 780
aagtacctcg aggacgtgga gatcggcacc gagttcggca ccccgccac cacctccgcc 840
cacctcgagt ggtggatcac caacatcacc ctcaccccgcc tcgaccgccc gctcatctcc 900
tag 903

```

<210> 57  
 <211> 387  
 <212> PRT  
 <213> *Thermus flavus*

```

<400> 57
Met Tyr Glu Pro Lys Pro Glu His Arg Phe Thr Phe Gly Leu Trp Thr
 1          5          10          15
Val Asp Asn Val Asp Arg Asp Pro Phe Gly Asp Thr Val Arg Glu Arg
 20          25          30
Leu Asp Pro Val Tyr Val Val His Lys Leu Ala Glu Leu Gly Ala Tyr
 35          40          45
Gly Val Asn Leu His Asp Glu Asp Leu Ile Pro Arg Gly Thr Pro Pro
 50          55          60
Gln Glu Arg Asp Gln Ile Val Arg Arg Phe Lys Lys Ala Leu Asp Glu
 65          70          75          80
Thr Val Leu Lys Val Pro Met Val Thr Ala Asn Leu Phe Ser Glu Pro
 85          90          95
Ala Phe Arg Asp Gly Ala Ser Thr Thr Arg Asp Pro Trp Val Trp Ala
 100          105          110
Tyr Ala Leu Arg Lys Ser Leu Glu Thr Met Asp Leu Gly Ala Glu Leu
 115          120          125
Gly Ala Glu Ile Tyr Met Phe Trp Met Val Arg Glu Arg Ser Glu Val
 130          135          140
Glu Ser Thr Asp Lys Thr Arg Lys Val Trp Asp Trp Val Arg Glu Thr
 145          150          155          160
Leu Asn Phe Met Thr Ala Tyr Thr Glu Asp Gln Gly Tyr Gly Tyr Arg
 165          170          175
Phe Ser Val Glu Pro Lys Pro Asn Glu Pro Arg Gly Asp Ile Tyr Phe
 180          185          190
Thr Thr Val Gly Ser Met Leu Ala Leu Ile His Thr Leu Asp Arg Pro
 195          200          205
Glu Arg Phe Gly Leu Asn Pro Glu Phe Ala His Glu Thr Met Ala Gly
 210          215          220
Leu Asn Phe Asp His Ala Val Ala Gln Ala Val Asp Ala Gly Lys Leu
 225          230          235          240
Phe His Ile Asp Leu Asn Asp Gln Arg Met Ser Arg Phe Asp Gln Asp
 245          250          255
Leu Arg Phe Gly Ser Glu Asn Leu Lys Ala Gly Phe Phe Leu Val Asp
 260          265          270
Leu Leu Glu Ser Ser Gly Tyr Gln Gly Pro Arg His Phe Glu Ala His
 275          280          285
Ala Leu Arg Thr Glu Asp Glu Glu Gly Val Trp Thr Phe Val Arg Val
 290          295          300
Cys Met Arg Thr Tyr Leu Ile Ile Lys Val Arg Ala Glu Thr Phe Arg
 305          310          315          320
Glu Asp Pro Glu Val Lys Glu Leu Leu Ala Ala Tyr Tyr Gln Glu Asp
 325          330          335
Pro Ala Thr Leu Ala Leu Leu Asp Pro Tyr Ser Arg Glu Lys Ala Glu

```

[illegible]

```
<210> 58
<211> 978
<212> DNA
<213> Artificial Sequence
```

```
<220>
<223> synthetic
```

<400>	58						
atgggggaaga	acggcaacct	gtgctgcttc	tctctgctgc	tgcttcttct	cgccggggttg	60	
gcgtccggcc	atcaaatcta	cttcgtggag	aagtaccaca	cctccgagga	caagtcacc	120	
tccaacacct	cctccacccc	gccgcagacc	accctctcca	ccaccaaggt	gctcaagatc	180	
cgctaccceg	acgacggtga	gtggcccggc	gccccgatcg	acaaggacgg	cgacggcaac	240	
ccggagttct	acatcgagat	caacctctgg	aacatcctca	acgccaccgg	cttcgccgag	300	
atgacctaca	acctcactag	tggcgtgctc	cactacgtgc	agcagctcga	caacatcgtg	360	
ctccgcgacc	gctccaactg	ggtgcacggc	tacccgaaa	tcttctacgg	caacaagccg	420	
tggaacgcca	actacgccac	cgacggcccg	atcccgcctc	cgccaaggt	gtccaacctc	480	
accgacttct	acctcaccat	ctcctacaag	ctcgagccga	agaacggtct	cccgatcaac	540	
ttcgccatcg	agtcttggt	caccgcgag	gcctggcgca	ccaccggcat	caactccgac	600	
gagcaggagg	tgatgatctg	gatctactac	gacggcctcc	agcccgcggg	ctccaaggtg	660	
aaggagatcg	tggtgccgat	catcgtgaac	ggcacccccg	tgaacgccac	cttcgaggtg	720	
tggaaggcca	acatcggctg	ggagtacgtg	gccttcgcga	tcaagacccc	gatcaaggag	780	
ggcaccgtga	ccatcccgtg	cggcgccttc	atctccgtgg	ccgccaacat	ctcctccctc	840	
ccgaactaca	ccgagaagta	cctcgaggac	gtggagatcg	gcaccgagtt	cggcaccccc	900	
tccaccacct	ccgcccacct	cgagtgggtg	atcaccaaca	tcacctcac	cccgctcgac	960	
cgcccgtcga	tctcctag					978	

```
<210> 59
<211> 1920
<212> DNA
<213> Aspergillus niger
```

<400> 59						
atgtccttcc	gctccctcct	cgccctctcc	ggcctcgtgt	gcaccggcct	cgccaacgtg	60
atctccaagc	gcgccaccct	cgactcctgg	ctctccaacg	aggccaccgt	ggcccgacc	120
gccatcctca	acaacatcgg	cgccgacggc	gcctgggtgt	ccggcgccga	ctccggcatc	180
gtggtggcct	ccccgtccac	cgacaacccg	gactacttct	acacctggac	ccgcgactcc	240
ggcctcgtgc	tcaagaccct	cgtggacctc	ttccgcaacg	gcgacacctc	cctcctctcc	300
accatcgaga	actacatctc	cgcccaggcc	atcgtgcagg	gcattctcaa	cccgtcgggc	360
gacctctcct	ccggcgccgg	cctcggcgag	ccgaagtcca	acgtggacga	gaccgcctac	420



```

accggctcct ggggcccggcc gcagcgcgac ggcccggccc tccgcgccac cgccatgatc 480
ggcttcggcc agtgggtcct cgacaacggc tacacctcca ccgccaccga catcgtgtgg 540
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&lt;210&gt; 60

&lt;211&gt; 6

30 &lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; synthetic

35

&lt;400&gt; 60

Ser Glu Lys Asp Glu Leu

1

5

40

&lt;210&gt; 61

&lt;211&gt; 561

&lt;212&gt; DNA

45 &lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Xylanase BD7436

50

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1) .. (561)

55

&lt;400&gt; 61

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 Met Ala Ser Thr Phe Tyr Trp His Leu Trp Thr Asp Gly Ile Gly Thr

48

1	5	10	15	
gtg aac gct acc aac ggc agc gac ggc aac tac agc gtg agc tgg agc				96
Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser				
5	20	25	30	
aac tgc ggc aac ttc gtg gtg ggc aag ggc tgg acc acc ggc agc gct				144
Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala				
10	35	40	45	
acc agg gtg atc aac tac aac gct cat gct ttc agc gtg gtg ggc aac				192
Thr Arg Val Ile Asn Tyr Asn Ala His Ala Phe Ser Val Val Gly Asn				
15	50	55	60	
gct tac ttg gct ttg tac ggc tgg acc agg aac agc ttg atc gag tac				240
Ala Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr				
20	65	70	75	
tac gtg gtg gac agc tgg ggc acc tac agg cca acc ggc acc tac aag				288
Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys				
25	85	90	95	
ggc acc gtg acc agc gac ggc ggc acc tac gac atc tac acc acc acc				336
Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr				
30	100	105	110	
agg acc aac gct cca agc atc gac ggc aac aac acc acc ttc acc caa				384
Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln				
35	115	120	125	
ttc tgg agc gtg agg caa agc aag agg cca atc ggc acc aac aac acc				432
Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr				
40	130	135	140	
atc acc ttc agc aac cat gtg aac gct tgg aag agc aag ggc atg aac				480
Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn				
45	145	150	155	
ttg ggc agc agc tgg agc tac caa gtg ttg gct acc gag ggc tac caa				528
Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu Ala Thr Glu Gly Tyr Gln				
50	165	170	175	
agc agc ggc tac agc aac gtg acc gtg tgg tag				561
Ser Ser Gly Tyr Ser Asn Val Thr Val Trp				
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<211> 186				
<212> PRT				
<213> Artificial Sequence				
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<223> Synthetic Construct				
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				20					25					30		
10	Asn	Cys	Gly	Asn	Phe	Val	Val	Gly	Lys	Gly	Trp	Thr	Thr	Gly	Ser	Ala
			35					40					45			
15	Thr	Arg	Val	Ile	Asn	Tyr	Asn	Ala	His	Ala	Phe	Ser	Val	Val	Gly	Asn
		50					55					60				
20	Ala	Tyr	Leu	Ala	Leu	Tyr	Gly	Trp	Thr	Arg	Asn	Ser	Leu	Ile	Glu	Tyr
	65					70					75					80
25	Tyr	Val	Val	Asp	Ser	Trp	Gly	Thr	Tyr	Arg	Pro	Thr	Gly	Thr	Tyr	Lys
					85					90					95	
30	Gly	Thr	Val	Thr	Ser	Asp	Gly	Gly	Thr	Tyr	Asp	Ile	Tyr	Thr	Thr	Thr
				100					105					110		
35	Arg	Thr	Asn	Ala	Pro	Ser	Ile	Asp	Gly	Asn	Asn	Thr	Thr	Phe	Thr	Gln
			115					120					125			
40	Phe	Trp	Ser	Val	Arg	Gln	Ser	Lys	Arg	Pro	Ile	Gly	Thr	Asn	Asn	Thr
		130					135					140				
45	Ile	Thr	Phe	Ser	Asn	His	Val	Asn	Ala	Trp	Lys	Ser	Lys	Gly	Met	Asn
	145					150					155					160
50	Leu	Gly	Ser	Ser	Trp	Ser	Tyr	Gln	Val	Leu	Ala	Thr	Glu	Gly	Tyr	Gln
					165					170					175	
55	Ser	Ser	Gly	Tyr	Ser	Asn	Val	Thr	Val	Trp						
				180					185							
	<210>	63														
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	<213>	Artificial Sequence														
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	<223>	Xylanase BD6002A														

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<220>
<221> CDS
<222> (1) .. (561)

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   Met Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Thr
   1          5          10          15

10  gtg aac gct acc aac ggc agc gac ggc aac tac agc gtg agc tgg agc 96
   Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser
           20          25          30

15  aac tgc ggc aac ttc gtg gtg ggc aag ggc tgg acc acc ggc agc gct 144
   Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala
           35          40          45

20  acc agg gtg atc aac tac aac gct ggc gct ttc agc cca agc ggc aac 192
   Thr Arg Val Ile Asn Tyr Asn Ala Gly Ala Phe Ser Pro Ser Gly Asn
           50          55          60

25  ggc tac ttg gct ttg tac ggc tgg acc agg aac agc ttg atc gag tac 240
   Gly Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr
   65          70          75          80

   tac gtg gtg gac agc tgg ggc acc tac agg cca acc ggc acc tac aag 288
   Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys
           85          90          95

30  ggc acc gtg acc agc gac ggc ggc acc tac gac atc tac acc acc acc 336
   Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr
           100          105          110

35  agg acc aac gct cca agc atc gac ggc aac aac acc acc ttc acc caa 384
   Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln
           115          120          125

40  ttc tgg agc gtg agg caa agc aag agg cca atc ggc acc aac aac acc 432
   Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr
           130          135          140

45  atc acc ttc agc aac cat gtg aac gct tgg aag agc aag ggc atg aac 480
   Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn
   145          150          155          160

   ttg ggc agc agc tgg agc tac caa gtg ttg gct acc gag ggc tac caa 528
   Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu Ala Thr Glu Gly Tyr Gln
           165          170          175

50  agc agc ggc tac agc aac gtg acc gtg tgg tag 561
   Ser Ser Gly Tyr Ser Asn Val Thr Val Trp
           180          185

55  <210> 64
     <211> 186
     <212> PRT

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Synthetic Construct

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&lt;400&gt; 64

Met Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Thr  
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 Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser  
 20 25 30  
 Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala  
 35 40 45  
 Thr Arg Val Ile Asn Tyr Asn Ala Gly Ala Phe Ser Pro Ser Gly Asn  
 50 55 60  
 Gly Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr  
 65 70 75 80  
 Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys  
 85 90 95  
 Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr  
 100 105 110  
 Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln  
 115 120 125  
 Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr  
 130 135 140  
 Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn  
 145 150 155 160  
 Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu Ala Thr Glu Gly Tyr Gln  
 165 170 175  
 Ser Ser Gly Tyr Ser Asn Val Thr Val Trp  
 180 185  
 <210> 65  
 <211> 561

<212> DNA  
 <213> Artificial Sequence

<220>

5 <223> Xylanase BD6002B

<220>

<221> CDS

10 <222> (1)..(561)

<400> 65

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	gtg aac gcc acc aac ggc tcc gac ggc aac tac tcc gtg tcc tgg tcc	96
	Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser	
	20 25 30	
20	aac tgc ggc aac ttc gtg gtg ggc aag ggc tgg acc acc ggc tcc gcc	144
	Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala	
	35 40 45	
25	acc cgc gtg atc aac tac aac gcc ggc gcc ttc tcc ccg tcc ggc aac	192
	Thr Arg Val Ile Asn Tyr Asn Ala Gly Ala Phe Ser Pro Ser Gly Asn	
	50 55 60	
30	ggc tac ctc gcc ctc tac ggc tgg acc cgc aac tcc ctc atc gag tac	240
	Gly Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr	
	65 70 75 80	
	tac gtg gtg gac tcc tgg ggc acc tac cgc ccg acc ggc acc tac aag	288
	Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys	
35	85 90 95	
	ggc acc gtg acc tcc gac ggc ggc acc tac gac atc tac acc acc acc	336
	Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr	
	100 105 110	
40	cgc acc aac gcc ccg tcc atc gac ggc aac aac acc acc ttc acc cag	384
	Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln	
	115 120 125	
45	ttc tgg tcc gtg cgc cag tcc aag cgc ccg atc ggc acc aac aac acc	432
	Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr	
	130 135 140	
50	atc acc ttc tcc aac cac gtg aac gcc tgg aag tcc aag ggc atg aac	480
	Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn	
	145 150 155 160	
	ctc ggc tcc tcc tgg tcc tac cag gtg ctc gcc acc gag ggc tac cag	528
	Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu Ala Thr Glu Gly Tyr Gln	
55	165 170 175	
	tcc tcc ggc tac tcc aac gtg acc gtg tgg tga	561



Ser Ser Gly Tyr Ser Asn Val Thr Val Trp  
180 185

5 <210> 66  
<211> 186  
<212> PRT  
<213> Artificial Sequence

10 <220>  
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15 Met Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Thr  
1 5 10 15

20 Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser  
20 25 30

25 Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala  
35 40 45

Thr Arg Val Ile Asn Tyr Asn Ala Gly Ala Phe Ser Pro Ser Gly Asn  
50 55 60

30 Gly Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr  
65 70 75 80

35 Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys  
85 90 95

40 Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr  
100 105 110

45 Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln  
115 120 125

Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr  
130 135 140

50 Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn  
145 150 155 160

55 Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu Ala Thr Glu Gly Tyr Gln  
165 170 175

Ser Ser Gly Tyr Ser Asn Val Thr Val Trp  
 180 185

5  
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 <213> Oryza sativa

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 15 <223> Promoter

<400> 67  
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 gtgaatgtgg tggcgtgttc agtgccttgg atttgagttt gatgagagtt gcttctgggt 360  
 30 caccactcac cattatcgat gctcctcttc agcataaggt aaaagtcttc cctgttttacg 420  
 ttattttacc cactatgggt gcttggggtg gttttttcct gattgcttat gccatggaaa 480  
 gtcatttgat atgttgaact tgaattaact gtagaattgt atacatgttc catttgtgtt 540  
 35 gtacttcctt cttttctatt agtagcctca gatgagtgtg aaaaaaacag attatataac 600  
 ttgccctata aatcatttga aaaaaatatt gtacagtgag aaattgatat atagtgaatt 660  
 40 tttaagagca tgttttccta aagaagtata tattttctat gtacaaaggc cattgaagta 720  
 attgtagata caggataatg tagacttttt ggacttacac tgctaccttt aagtaacaat 780  
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 gaacacagcc ttaaccacgc caaataatgc tacaacctac cagtccacac ctcttgtaaa 1080  
 gcatttggtg catggaaaag ctaagatgac agcaacctgt tcaggaaaac aactgacaag 1140  
 55 gtcataggga gagggagctt ttggaaaggt gccgtgcagt tcaaacaatt agttagcagt 1200

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5 taatgaaaga agatgtggtg ttagaaaagg aaacaatatc atgagtaatg tgtgggcatt 1380
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25 acacttcata tatcatgagt cacttcatgt ctggacatta acaaactcta tcttaacatt 1980
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30

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&lt;210&gt; 68

&lt;211&gt; 79

&lt;212&gt; PRT

35 &lt;213&gt; Zea mays

&lt;220&gt;

&lt;221&gt; SIGNAL

40 &lt;222&gt; (1)..(79)

&lt;223&gt; Maize waxy signal sequence.

&lt;400&gt; 68

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45 Met Leu Ala Ala Leu Ala Thr Ser Gln Leu Val Ala Thr Arg Ala Gly
   1             5             10             15

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50 Leu Gly Val Pro Asp Ala Ser Thr Phe Arg Arg Gly Ala Ala Gln Gly
   20             25             30

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55 Leu Arg Gly Ala Arg Ala Ser Ala Ala Ala Asp Thr Leu Ser Met Arg
   35             40             45

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Thr Ser Ala Arg Ala Ala Pro Arg His Gln His Gln Gln Ala Arg Arg

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30	atg tgg gcc tcc ccg tcc gcc gcc tcc gcg gac gag ccg tcc gac ccg Met Trp Ala Ser Pro Ser Ala Ala Ser Ala Asp Glu Pro Ser Asp Pro 20 25 30					96
35	atg atg aag cgc ttc gag gag tgg atg gtg gag tac ggc cgc gtg tac Met Met Lys Arg Phe Glu Glu Trp Met Val Glu Tyr Gly Arg Val Tyr 35 40 45					144
40	aag gac aac gac gag aag atg cgc cgc ttc cag atc ttc aag aac aac Lys Asp Asn Asp Glu Lys Met Arg Arg Phe Gln Ile Phe Lys Asn Asn 50 55 60					192
45	gtg aac cac atc gag acc ttc aac tcc cgc aac gag aac tcc tac acc Val Asn His Ile Glu Thr Phe Asn Ser Arg Asn Glu Asn Ser Tyr Thr 65 70 75 80					240
50	ctc ggc atc aac cag ttc acc gac atg acc aac aac gag ttc atc gcc Leu Gly Ile Asn Gln Phe Thr Asp Met Thr Asn Asn Glu Phe Ile Ala 85 90 95					288
55	cag tac acc ggc ggc atc tcc cgc ccg ctc aac atc gag cgc gag ccg Gln Tyr Thr Gly Gly Ile Ser Arg Pro Leu Asn Ile Glu Arg Glu Pro 100 105 110					336
60	gtg gtg tcc ttc gac gac gtg gac atc tcc gcc gtg ccg cag tcc atc Val Val Ser Phe Asp Asp Val Asp Ile Ser Ala Val Pro Gln Ser Ile 115 120 125					384
65	gac tgg cgc gac tac ggc gcc gtg acc tcc gtg aag aac cag aac ccg Asp Trp Arg Asp Tyr Gly Ala Val Thr Ser Val Lys Asn Gln Asn Pro 130 135 140					432

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	145					150					155					160	
5																	
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	Tyr	Lys	Ile	Lys	Lys	Gly	Ile	Leu	Glu	Pro	Leu	Ser	Glu	Gln	Gln	Val	
					165					170					175		
10																	
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15																	
	gcc	ttc	gag	ttc	atc	atc	tcc	aac	aag	ggc	gtg	gcc	tcc	ggc	gcc	atc	624
	Ala	Phe	Glu	Phe	Ile	Ile	Ser	Asn	Lys	Gly	Val	Ala	Ser	Gly	Ala	Ile	
			195					200					205				
20																	
	tac	ccg	tac	aag	gcc	gcc	aag	ggc	acc	tgc	aag	acc	gac	ggc	gtg	ccg	672
	Tyr	Pro	Tyr	Lys	Ala	Ala	Lys	Gly	Thr	Cys	Lys	Thr	Asp	Gly	Val	Pro	
		210					215					220					
25																	
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	Asn	Ser	Ala	Tyr	Ile	Thr	Gly	Tyr	Ala	Arg	Val	Pro	Arg	Asn	Asn	Glu	
	225					230					235					240	
25																	
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	Ser	Ser	Met	Met	Tyr	Ala	Val	Ser	Lys	Gln	Pro	Ile	Thr	Val	Ala	Val	
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30																	
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	Pro Ser Gly Gly Asn Pro Pro Gly Gly Asn Pro Pro Gly Thr Thr Thr	
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	Thr Arg Arg Pro Ala Thr Thr Thr Gly Ser Ser Pro Gly Pro Thr Gln	
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	Ser His Tyr Gly Gln Cys Gly Gly Ile Gly Tyr Ser Gly Pro Thr Val	
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	Ser Thr Tyr Gly Val Thr Thr Ser Gly Asn Ser Leu Ser Ile Gly Phe	
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	Val Thr Gln Ser Ala Gln Lys Asn Val Gly Ala Arg Leu Tyr Leu Met	

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15	Leu Tyr Phe Val Ser Met Asp Ala Asp Gly Gly Val Ser Lys Tyr Pro 145 150 155 160		
20	Thr Asn Thr Ala Gly Ala Lys Tyr Gly Thr Gly Tyr Cys Asp Ser Gln 165 170 175		
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	Cys	Gly	Gly	Gln	Asn	Trp	Ser	Gly	Pro	Thr	Cys	Cys	Ala	Ser	Gly	Ser	
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	Thr	Cys	Val	Tyr	Ser	Asn	Asp	Tyr	Tyr	Ser	Gln	Cys	Leu	Pro	Gly	Ala	
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	Thr	Thr	Thr	Arg	Val	Pro	Pro	Val	Gly	Ser	Gly	Thr	Ala	Thr	Tyr	Ser	
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	Ser	Glu	Val	Ser	Ser	Leu	Ala	Ile	Pro	Ser	Leu	Thr	Gly	Ala	Met	Ala	
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40	Thr	Leu	Asp	Lys	Thr	Pro	Leu	Met	Glu	Gln	Thr	Leu	Ala	Asp	Ile	Arg	
	145					150					155				160		
	acc	gcc	aac	aag	aat	ggc	ggg	aac	tat	gcc	gga	cag	ttt	gtg	gtg	tat	528
	Thr	Ala	Asn	Lys	Asn	Gly	Gly	Asn	Tyr	Ala	Gly	Gln	Phe	Val	Val	Tyr	
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45	gac	ttg	ccg	gat	cgc	gat	tgc	gct	gcc	ctt	gcc	tcg	aat	ggc	gaa	tac	576
	Asp	Leu	Pro	Asp	Arg	Asp	Cys	Ala	Ala	Leu	Ala	Ser	Asn	Gly	Glu	Tyr	
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	Ser	Ile	Ala	Asp	Gly	Gly	Val	Ala	Lys	Tyr	Lys	Asn	Tyr	Ile	Asp	Thr	
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	Val	Thr	Gln	Leu	Asn	Leu	Pro	Asn	Val	Ala	Met	Tyr	Leu	Asp	Ala	Gly	
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	His	Ala	Gly	Trp	Leu	Gly	Trp	Pro	Ala	Asn	Gln	Asp	Pro	Ala	Ala	Gln	
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	Leu	Phe	Ala	Asn	Val	Tyr	Lys	Asn	Ala	Ser	Ser	Pro	Arg	Ala	Leu	Arg	
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30	ccc	cca	tcg	tac	acg	caa	ggc	aac	gct	gtc	tac	aac	gag	aag	ctg	tac	1008
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	Gln	Gln	Trp	Gly	Asp	Trp	Cys	Asn	Val	Ile	Gly	Thr	Gly	Phe	Gly	Ile	
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	Val	Lys	Pro	Gly	Gly	Glu	Cys	Asp	Gly	Thr	Ser	Asp	Ser	Ser	Ala	Pro	
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	Arg	Phe	Asp	Ser	His	Cys	Ala	Leu	Pro	Asp	Ala	Leu	Gln	Pro	Ala	Pro	
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	Gln	Ala	Gly	Ala	Trp	Phe	Gln	Ala	Tyr	Phe	Val	Gln	Leu	Leu	Thr	Asn	
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	Lys Tyr Gln Gln Asn Gly Val Asp Ile Pro Ser Ala Gln Pro Gly Gly	
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55	Ala	Thr	Cys	Gly	Lys	Asn	Cys	Phe	Ile	Glu	Gly	Val	Asp	Tyr	Ala	Ala	
	65				70					75					80		
60	Ser	Gly	Val	Thr	Thr	Ser	Gly	Ser	Ser	Leu	Thr	Met	Asn	Gln	Tyr	Met	
				85						90					95		
65	Pro	Ser	Ser	Ser	Gly	Gly	Tyr	Ser	Ser	Val	Ser	Pro	Arg	Leu	Tyr	Leu	
				100				105						110			



5      Leu Asp Ser Asp Gly Glu Tyr Val Met Leu Lys Leu Asn Gly Gln Glu  
               115                               120                               125

10      Leu Ser Phe Asp Val Asp Leu Ser Ala Leu Pro Cys Gly Glu Asn Gly  
               130                               135                               140

15      Ser Leu Tyr Leu Ser Gln Met Asp Glu Asn Gly Gly Ala Asn Gln Tyr  
               145                               150                               155                               160

20      Asn Thr Ala Gly Ala Asn Tyr Gly Ser Gly Tyr Cys Asp Ala Gln Cys  
                               165                               170                               175

25      Pro Val Gln Thr Trp Arg Asn Gly Thr Leu Asn Thr Ser His Gln Gly  
                               180                               185                               190

30      Phe Cys Cys Asn Glu Met Asp Ile Leu Glu Gly Asn Ser Arg Ala Asn  
                               195                               200                               205

35      Ala Leu Thr Pro His Ser Cys Thr Ala Thr Ala Cys Asp Ser Ala Gly  
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40      Cys Gly Phe Asn Pro Tyr Gly Ser Gly Tyr Lys Ser Tyr Tyr Gly Pro  
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45      Gly Asp Thr Val Asp Thr Ser Lys Thr Phe Thr Ile Ile Thr Gln Phe  
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50      Asn Thr Asp Asn Gly Ser Pro Ser Gly Asn Leu Val Ser Ile Thr Arg  
                               260                               265                               270

55      Lys Tyr Gln Gln Asn Gly Val Asp Ile Pro Ser Ala Gln Pro Gly Gly  
                               275                               280                               285

60      Asp Thr Ile Ser Ser Cys Pro Ser Ala Ser Ala Tyr Gly Gly Leu Ala  
               290                               295                               300

65      Thr Met Gly Lys Ala Leu Ser Ser Gly Met Val Leu Val Phe Ser Ile  
               305                               310                               315                               320

70      Trp Asn Asp Asn Ser Gln Tyr Met Asn Trp Leu Asp Ser Gly Asn Ala  
                               325                               330                               335

Gly Pro Cys Ser Ser Thr Glu Gly Asn Pro Ser Asn Thr Leu Ala Asn  
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Asn Pro Asn Thr His Val Val Phe Ser Asn Ile Arg Trp Gly Asp Ile  
 355 360 365

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Gly Ser Thr Thr Asn Ser Thr Ala Pro Pro Pro Pro Pro Ala Ser Ser  
 370 375 380

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Thr Thr Phe Ser Thr Thr Arg Arg Ser Ser Thr Thr Ser Ser Ser Pro  
 385 390 395 400

20

Ser Cys Thr Gln Thr His Trp Gly Gln Cys Gly Gly Ile Gly Tyr Ser  
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aac atc ggc aac gcc ctg gag gcc ccg aac gag ggc gac tgg ggc gtg 96  
 Asn Ile Gly Asn Ala Leu Glu Ala Pro Asn Glu Gly Asp Trp Gly Val  
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gtg atc aag gac gag ttc ttc gac atc atc aag gag gcc ggc ttc tcc 144  
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cac gtg cgc atc ccg atc cgc tgg tcc acc cac gcc tac gcc ttc ccg 192

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	Pro	Tyr	Lys	Ile	Met	Asp	Arg	Phe	Phe	Lys	Arg	Val	Asp	Glu	Val	Ile	
	65					70					75					80	
10	aac	ggc	gcc	ctc	aag	cgc	ggc	ctc	gcc	gtg	gcc	atc	aac	atc	cac	cac	288
	Asn	Gly	Ala	Leu	Lys	Arg	Gly	Leu	Ala	Val	Ala	Ile	Asn	Ile	His	His	
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15	tac	gag	gag	ctc	atg	aac	gac	ccg	gag	gag	cac	aag	gag	cgc	ttc	ctc	336
	Tyr	Glu	Glu	Leu	Met	Asn	Asp	Pro	Glu	Glu	His	Lys	Glu	Arg	Phe	Leu	
				100					105					110			
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	Ala	Leu	Trp	Lys	Gln	Ile	Ala	Asp	Arg	Tyr	Lys	Asp	Tyr	Pro	Glu	Thr	
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25	ctc	ttc	ttc	gag	atc	ctc	aac	gag	ccg	cac	ggc	aac	ctc	acc	ccg	gag	432
	Leu	Phe	Phe	Glu	Ile	Leu	Asn	Glu	Pro	His	Gly	Asn	Leu	Thr	Pro	Glu	
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	Lys	Trp	Asn	Glu	Leu	Leu	Glu	Glu	Ala	Leu	Lys	Val	Ile	Arg	Ser	Ile	
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	Asp	Lys	Lys	His	Thr	Ile	Ile	Ile	Gly	Thr	Ala	Glu	Trp	Gly	Gly	Ile	
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	Ser	Ala	Leu	Glu	Lys	Leu	Ser	Val	Pro	Lys	Trp	Glu	Lys	Asn	Ser	Ile	
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	Val	Thr	Ile	His	Tyr	Tyr	Asn	Pro	Phe	Glu	Phe	Thr	His	Gln	Gly	Ala	
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	Glu	Trp	Val	Glu	Gly	Ser	Glu	Lys	Trp	Leu	Gly	Arg	Lys	Trp	Gly	Ser	
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	Pro	Asp	Asp	Gln	Lys	His	Leu	Ile	Glu	Glu	Phe	Asn	Phe	Ile	Glu	Glu	
	225					230					235					240	
60	tgg	tcc	aag	aag	aac	aag	cgc	ccg	atc	tac	atc	ggc	gag	ttt	ggc	gcc	768
	Trp	Ser	Lys	Lys	Asn	Lys	Arg	Pro	Ile	Tyr	Ile	Gly	Glu	Phe	Gly	Ala	
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65	tac	cgc	aag	gcc	gac	ctc	gag	tcc	cgc	atc	aag	tgg	acc	tcc	ttc	gtg	816
	Tyr	Arg	Lys	Ala	Asp	Leu	Glu	Ser	Arg	Ile	Lys	Trp	Thr	Ser	Phe	Val	
				260					265					270			
70	gtg	cgt	gag	atg	gag	aag	cgc	cgc	tgg	tcc	tgg	gcc	tac	tgg	gag	ttc	864
	Val	Arg	Glu	Met	Glu	Lys	Arg	Arg	Trp	Ser	Trp	Ala	Tyr	Trp	Glu	Phe	

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	Cys Ser Gly Phe Gly Val Tyr Asp Thr Leu Arg Lys Thr Trp Asn Lys			
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	35 40 45			
35	His Val Arg Ile Pro Ile Arg Trp Ser Thr His Ala Tyr Ala Phe Pro			
	50 55 60			
40	Pro Tyr Lys Ile Met Asp Arg Phe Phe Lys Arg Val Asp Glu Val Ile			
	65 70 75 80			
45	Asn Gly Ala Leu Lys Arg Gly Leu Ala Val Ala Ile Asn Ile His His			
	85 90 95			
50	Tyr Glu Glu Leu Met Asn Asp Pro Glu Glu His Lys Glu Arg Phe Leu			
	100 105 110			
55	Ala Leu Trp Lys Gln Ile Ala Asp Arg Tyr Lys Asp Tyr Pro Glu Thr			
	115 120 125			
55	Leu Phe Phe Glu Ile Leu Asn Glu Pro His Gly Asn Leu Thr Pro Glu			
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Lys Trp Asn Glu Leu Leu Glu Glu Ala Leu Lys Val Ile Arg Ser Ile  
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5 Asp Lys Lys His Thr Ile Ile Ile Gly Thr Ala Glu Trp Gly Gly Ile  
 165 170 175

10 Ser Ala Leu Glu Lys Leu Ser Val Pro Lys Trp Glu Lys Asn Ser Ile  
 180 185 190

15 Val Thr Ile His Tyr Tyr Asn Pro Phe Glu Phe Thr His Gln Gly Ala  
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Glu Trp Val Glu Gly Ser Glu Lys Trp Leu Gly Arg Lys Trp Gly Ser  
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20 Pro Asp Asp Gln Lys His Leu Ile Glu Glu Phe Asn Phe Ile Glu Glu  
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25 Trp Ser Lys Lys Asn Lys Arg Pro Ile Tyr Ile Gly Glu Phe Gly Ala  
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30 Tyr Arg Lys Ala Asp Leu Glu Ser Arg Ile Lys Trp Thr Ser Phe Val  
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35 Val Arg Glu Met Glu Lys Arg Arg Trp Ser Trp Ala Tyr Trp Glu Phe  
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Cys Ser Gly Phe Gly Val Tyr Asp Thr Leu Arg Lys Thr Trp Asn Lys  
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10	gcc Ala	gct Ala	gcc Ala 35	gga Gly	gtc Val	acc Thr	cac His	gtc Val 40	tgg Trp	ctg Leu	cca Pro	ccg Pro	ccg Pro 45	tcg Ser	cac His	tcc Ser	144
15	gtc Val	tcc Ser 50	aac Asn	gaa Glu	ggg Gly	tac Tyr	atg Met 55	cct Pro	ggg Gly	cgg Arg	ctg Leu	tac Tyr 60	gac Asp	atc Ile	gac Asp	gcg Ala	192
20	tcc Ser 65	aag Lys	tac Tyr	ggc Gly	aac Asn 70	gcg Ala	gcg Ala	gag Glu	ctc Leu	aag Lys	tcg Ser 75	ctc Leu	atc Ile	ggc Gly	gcg Ala	ctc Leu 80	240
25	cac His	ggc Gly	aag Lys	ggc Gly	gtg Val 85	cag Gln	gcc Ala	atc Ile	gcc Ala 90	gac Asp	atc Ile	gtc Val	atc Ile	aac Asn 95	cac His	cg Arg	288
30	tgc Cys	gcc Ala	gac Asp	tac Tyr 100	aag Lys	gat Asp	agc Ser	cgc Arg	ggc Gly 105	atc Ile	tac Tyr	tgc Cys	atc Ile	ttc Phe 110	gag Glu	ggc Gly	336
35	ggc Gly	acc Thr	tcc Ser 115	gac Asp	ggc Gly	cgc Arg	ctc Leu	gac Asp 120	tgg Trp	ggc Gly	ccc Pro	cac His	atg Met 125	atc Ile	tgt Cys	cg Arg	384
40	gac Asp 130	gac Asp	acc Thr	aaa Lys	tac Tyr	tcc Ser	gat Asp 135	ggc Gly	acc Thr	gca Ala	aac Asn	ctc Leu 140	gac Asp	acc Thr	gga Gly	gcc Ala	432
45	gac Asp 145	ttc Phe	gcc Ala	gcc Ala	gcg Ala 150	ccc Pro	gac Asp	atc Ile	gac Asp	cac His	ctc Leu 155	aac Asn	gac Asp	cg Arg	gtc Val	cag Gln 160	480
50	cg Arg	gag Glu	ctc Leu	aag Lys	gag Glu 165	tgg Trp	ctc Leu	tgg Trp 170	ctc Leu	aag Lys	agc Ser	gac Asp	ctc Leu	ggc Gly 175	ttc Phe		528
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65	tgg Trp 210	gac Asp	aat Asn	atg Met	gcc Ala	acc Thr	ggc Gly 215	ggc Gly	gac Asp	ggc Gly	aag Lys	ccc Pro 220	aac Asn	tac Tyr	gac Asp	cag Gln	672
70	gac Asp	gag Ala	cac His	cg Arg	cag Gln	aat Asn	ctg Leu	gtg Val	aac Asn	tgg Trp	gtg Val	gac Asp	aag Lys	gtg Val	ggc Gly	ggc Gly	720



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10	aac gct gcc gtg gag ggc gag ctg tgg agg ctg atc gac ccg cag ggg Asn Ala Ala Val Glu Gly Glu Leu Trp Arg Leu Ile Asp Pro Gln Gly 260 265 270							816
15	aag gcc ccc ggc gtg atg gga tgg tgg ccg gcc aag gcc gtc acc ttc Lys Ala Pro Gly Val Met Gly Trp Trp Pro Ala Lys Ala Val Thr Phe 275 280 285							864
20	gtc gac aac cac gat aca ggc tcc acg cag gcc atg tgg cca ttc ccc Val Asp Asn His Asp Thr Gly Ser Thr Gln Ala Met Trp Pro Phe Pro 290 295 300							912
25	tcc gac aag gtc atg cag ggc tac gcg tac atc ctc acc cac ccc ggc Ser Asp Lys Val Met Gln Gly Tyr Ala Tyr Ile Leu Thr His Pro Gly 305 310 315 320							960
30	atc cca tgc atc ttc tac gac cat ttc ttc aac tgg ggg ttt aag gac Ile Pro Cys Ile Phe Tyr Asp His Phe Phe Asn Trp Gly Phe Lys Asp 325 330 335							1008
35	cag atc gcg gcg ctg gtg gcg atc agg aag cgc aac ggc atc acg gcg Gln Ile Ala Ala Leu Val Ala Ile Arg Lys Arg Asn Gly Ile Thr Ala 340 345 350							1056
40	acg agc gct ctg aag atc ctc atg cac gaa gga gat gcc tac gtc gcc Thr Ser Ala Leu Lys Ile Leu Met His Glu Gly Asp Ala Tyr Val Ala 355 360 365							1104
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50	ggg gcg gtg atc ccg gcc ggg ttc gtg acc tcg gca cac ggc aac gac Gly Ala Val Ile Pro Ala Gly Phe Val Thr Ser Ala His Gly Asn Asp 385 390 395 400							1200
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 Ala Ala Ala Gly Val Thr His Val Trp Leu Pro Pro Pro Ser His Ser  
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 10 Val Ser Asn Glu Gly Tyr Met Pro Gly Arg Leu Tyr Asp Ile Asp Ala  
                     50                    55                    60  
 15 Ser Lys Tyr Gly Asn Ala Ala Glu Leu Lys Ser Leu Ile Gly Ala Leu  
                     65                    70                    75                    80  
 His Gly Lys Gly Val Gln Ala Ile Ala Asp Ile Val Ile Asn His Arg  
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 Cys Ala Asp Tyr Lys Asp Ser Arg Gly Ile Tyr Cys Ile Phe Glu Gly  
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 25 Gly Thr Ser Asp Gly Arg Leu Asp Trp Gly Pro His Met Ile Cys Arg  
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 30 Asp Asp Thr Lys Tyr Ser Asp Gly Thr Ala Asn Leu Asp Thr Gly Ala  
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 35 Asp Phe Ala Ala Ala Pro Asp Ile Asp His Leu Asn Asp Arg Val Gln  
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 Asp Ala Trp Arg Leu Asp Phe Ala Arg Gly Tyr Ser Pro Glu Met Ala  
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5 Asn Ala Ala Val Glu Gly Glu Leu Trp Arg Leu Ile Asp Pro Gln Gly  
260 265 270

10 Lys Ala Pro Gly Val Met Gly Trp Trp Pro Ala Lys Ala Val Thr Phe  
275 280 285

15 Val Asp Asn His Asp Thr Gly Ser Thr Gln Ala Met Trp Pro Phe Pro  
290 295 300

Ser Asp Lys Val Met Gln Gly Tyr Ala Tyr Ile Leu Thr His Pro Gly  
305 310 315 320

20 Ile Pro Cys Ile Phe Tyr Asp His Phe Phe Asn Trp Gly Phe Lys Asp  
325 330 335

25 Gln Ile Ala Ala Leu Val Ala Ile Arg Lys Arg Asn Gly Ile Thr Ala  
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30 Thr Ser Ala Leu Lys Ile Leu Met His Glu Gly Asp Ala Tyr Val Ala  
355 360 365

35 Glu Ile Asp Gly Lys Val Val Val Lys Ile Gly Ser Arg Tyr Asp Val  
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	Ile	Glu	Gly	Ala	Val	Asp	Gln	Asp	Gly	Arg	Gly	Pro	Ser	Ile	Trp	Asp	
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	Thr	Phe	Cys	Ala	Gln	Pro	Gly	Lys	Ile	Ala	Asp	Gly	Ser	Ser	Gly	Val	
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20	acg	gcg	tgc	gac	tcg	tac	aac	cgc	acg	gcc	gag	gac	att	gcg	ctg	ctg	192
	Thr	Ala	Cys	Asp	Ser	Tyr	Asn	Arg	Thr	Ala	Glu	Asp	Ile	Ala	Leu	Leu	
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25	aag	tcg	ctc	ggg	gcc	aag	agc	tac	cgc	ttc	tcc	atc	tcg	tgg	tcg	cgc	240
	Lys	Ser	Leu	Gly	Ala	Lys	Ser	Tyr	Arg	Phe	Ser	Ile	Ser	Trp	Ser	Arg	
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35	gac	cac	tac	gtc	aag	ttc	gtc	gac	gac	ctg	ctc	gac	gcc	ggc	atc	acg	336
	Asp	His	Tyr	Val	Lys	Phe	Val	Asp	Asp	Leu	Leu	Asp	Ala	Gly	Ile	Thr	
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	Arg	Tyr	Gly	Gly	Leu	Leu	Asn	Arg	Thr	Glu	Phe	Pro	Leu	Asp	Phe	Glu	
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		210					215					220					

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	gag Glu	gcg Ala	gcc Ala	gag Glu	cgg Arg 245	cgc Arg	ctc Leu	gag Glu	ttc Phe	ttc Phe 250	acg Thr	gcc Ala	tgg Trp	ttc Phe	gcg Ala 255	gac Asp	768
	ccc Pro	atc Ile	tac Tyr	ttg Leu 260	ggc Gly	gac Asp	tac Tyr	ccg Pro	gcg Ala 265	tcg Ser	atg Met	cgc Arg	aag Lys	cag Gln 270	ctg Leu	ggc Gly	816
15	gac Asp	cgg Arg	ctg Leu 275	ccg Pro	acc Thr	ttt Phe	acg Thr	ccc Pro 280	gag Glu	gag Glu	cgc Arg	gcc Ala	ctc Leu 285	gtc Val	cac His	ggc Gly	864
20	tcc Ser	aac Asn 290	gac Asp	ttt Phe	tac Tyr	ggc Gly	atg Met 295	aac Asn	cac His	tac Tyr	acg Thr	tcc Ser 300	aac Asn	tac Tyr	atc Ile	cgc Arg	912
25	cac His 305	cgc Arg	agc Ser	tcg Ser	ccc Pro	gcc Ala 310	tcc Ser	gcc Ala	gac Asp	gac Asp	acc Thr 315	gtc Val	ggc Gly	aac Asn	gtc Val	gac Asp 320	960
30	gtg Val	ctc Leu	ttc Phe	acc Thr	aac Asn 325	aag Lys	cag Gln	ggc Gly	aac Asn	tgc Cys 330	atc Ile	ggc Gly	ccc Pro	gag Glu	acg Thr 335	cag Gln	1008
35	tcc Ser	ccc Pro	tgg Trp	ctg Leu 340	cgc Arg	ccc Pro	tgt Cys	gcc Ala	gcc Ala 345	ggc Gly	ttc Phe	cgc Arg	gac Asp	ttc Phe 350	ctg Leu	gtg Val	1056
40	tgg Trp	atc Ile	agc Ser 355	aag Lys	agg Arg	tac Tyr	ggc Gly	tac Tyr	ccg Pro	ccc Pro	atc Ile	tac Tyr	gtg Val 365	acg Thr	gag Glu	aac Asn	1104
45	ggc Gly	acg Thr 370	agc Ser	atc Ile	aag Lys	ggc Gly	gag Glu 375	agc Ser	gac Asp	ttg Leu	ccc Pro	aag Lys 380	gag Glu	aag Lys	att Ile	ctc Leu	1152
50	gaa Glu 385	gat Asp	gac Asp	ttc Phe	agg Arg	gtc Val 390	aag Lys	tac Tyr	tat Tyr	aac Asn	gag Glu 395	tac Tyr	atc Ile	cgt Arg	gcc Ala	atg Met 400	1200
55	gtt Val	acc Thr	gcc Ala	gtg Val	gag Glu 405	ctg Leu	gac Asp	ggg Gly	gtc Val	aac Asn 410	gtc Val	aag Lys	ggg Gly	tac Tyr	ttt Phe 415	gcc Ala	1248
60	tgg Trp	tcg Ser	ctc Leu	atg Met 420	gac Asp	aac Asn	ttt Phe	gag Glu	tgg Trp 425	gcg Ala	gac Asp	ggc Gly	tac Tyr	gtg Val 430	acg Thr	agg Arg	1296
65	ttt Phe	ggg Gly	gtt Val	acg Thr	tat Tyr	gtg Val	gat Asp	tat Tyr	gag Glu	aat Asn	ggg Gly	cag Gln	aag Lys 445	cgg Arg	ttc Phe	ccc Pro	1344

aag aag agc gca aag agc ttg aag ccg ctg ttt gac gag ctg att gcg 1392  
 Lys Lys Ser Ala Lys Ser Leu Lys Pro Leu Phe Asp Glu Leu Ile Ala  
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5 gcg gcg tga 1401  
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30 Thr Phe Cys Ala Gln Pro Gly Lys Ile Ala Asp Gly Ser Ser Gly Val  
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35 Thr Ala Cys Asp Ser Tyr Asn Arg Thr Ala Glu Asp Ile Ala Leu Leu  
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40 Lys Ser Leu Gly Ala Lys Ser Tyr Arg Phe Ser Ile Ser Trp Ser Arg  
 65 70 75 80

45 Ile Ile Pro Glu Gly Gly Arg Gly Asp Ala Val Asn Gln Ala Gly Ile  
 85 90 95

50 Asp His Tyr Val Lys Phe Val Asp Asp Leu Leu Asp Ala Gly Ile Thr  
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55 Pro Phe Ile Thr Leu Phe His Trp Asp Leu Pro Glu Gly Leu His Gln  
 115 120 125

60 Arg Tyr Gly Gly Leu Leu Asn Arg Thr Glu Phe Pro Leu Asp Phe Glu  
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65 Asn Tyr Ala Arg Val Met Phe Arg Ala Leu Pro Lys Val Arg Asn Trp  
 145 150 155 160



Ile Thr Phe Asn Glu Pro Leu Cys Ser Ala Ile Pro Gly Tyr Gly Ser  
165 170 175  
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Gly Thr Phe Ala Pro Gly Arg Gln Ser Thr Ser Glu Pro Trp Thr Val  
180 185 190

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Gly His Asn Ile Leu Val Ala His Gly Arg Ala Val Lys Ala Tyr Arg  
195 200 205

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Asp Asp Phe Lys Pro Ala Ser Gly Asp Gly Gln Ile Gly Ile Val Leu  
210 215 220

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Asn Gly Asp Phe Thr Tyr Pro Trp Asp Ala Ala Asp Pro Ala Asp Lys  
225 230 235 240

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Glu Ala Ala Glu Arg Arg Leu Glu Phe Phe Thr Ala Trp Phe Ala Asp  
245 250 255

30

Pro Ile Tyr Leu Gly Asp Tyr Pro Ala Ser Met Arg Lys Gln Leu Gly  
260 265 270

35

Asp Arg Leu Pro Thr Phe Thr Pro Glu Glu Arg Ala Leu Val His Gly  
275 280 285

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Ser Asn Asp Phe Tyr Gly Met Asn His Tyr Thr Ser Asn Tyr Ile Arg  
290 295 300

45

His Arg Ser Ser Pro Ala Ser Ala Asp Asp Thr Val Gly Asn Val Asp  
305 310 315 320

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Val Leu Phe Thr Asn Lys Gln Gly Asn Cys Ile Gly Pro Glu Thr Gln  
325 330 335

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Ser Pro Trp Leu Arg Pro Cys Ala Ala Gly Phe Arg Asp Phe Leu Val  
340 345 350

Trp Ile Ser Lys Arg Tyr Gly Tyr Pro Pro Ile Tyr Val Thr Glu Asn  
355 360 365

Gly Thr Ser Ile Lys Gly Glu Ser Asp Leu Pro Lys Glu Lys Ile Leu  
370 375 380

Glu Asp Asp Phe Arg Val Lys Tyr Tyr Asn Glu Tyr Ile Arg Ala Met  
 385 390 395 400

5 Val Thr Ala Val Glu Leu Asp Gly Val Asn Val Lys Gly Tyr Phe Ala  
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10 Trp Ser Leu Met Asp Asn Phe Glu Trp Ala Asp Gly Tyr Val Thr Arg  
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15 Phe Gly Val Thr Tyr Val Asp Tyr Glu Asn Gly Gln Lys Arg Phe Pro  
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20 Lys Lys Ser Ala Lys Ser Leu Lys Pro Leu Phe Asp Glu Leu Ile Ala  
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Ala Ala  
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 Phe Val Ala Asn Asp Gln Glu His Glu Arg Arg Ala Val Asp Cys Leu  
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 Ile Thr Gln Arg Ala Leu Arg Glu Val Tyr Leu Arg Pro Phe Gln Ile  
 35 40 45

55 gta gcc cga gat gca agg ccc ggc gca ttg atg aca tcc tac aac aag 192  
 Val Ala Arg Asp Ala Arg Pro Gly Ala Leu Met Thr Ser Tyr Asn Lys  
 50 55 60

gtc aat ggc aag cac gtc gct gac agc gcc gag ttc ctt cag ggc att 240

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	Leu	Arg	Thr	Glu	Trp	Asn	Trp	Asp	Pro	Leu	Ile	Val	Ser	Asp	Trp	Tyr	
					85					90					95		
10	ggc	acc	tac	acc	act	att	gat	gcc	atc	aaa	gcc	ggc	ctt	gat	ctc	gag	336
	Gly	Thr	Tyr	Thr	Thr	Ile	Asp	Ala	Ile	Lys	Ala	Gly	Leu	Asp	Leu	Glu	
				100					105					110			
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	Met	Pro	Gly	Val	Ser	Arg	Tyr	Arg	Gly	Lys	Tyr	Ile	Glu	Ser	Ala	Leu	
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	Gln	Ala	Arg	Leu	Leu	Lys	Gln	Ser	Thr	Ile	Asp	Glu	Arg	Ala	Arg	Arg	
		130					135					140					
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	Glu	Gln	Gly	Arg	Asp	Phe	Pro	Glu	Asp	Arg	Val	Leu	Asn	Arg	Gln	Ile	
					165				170						175		
35	tgc	ggc	agc	agc	att	gtc	cta	ctg	aag	aat	gag	aac	tcc	atc	tta	cct	576
	Cys	Gly	Ser	Ser	Ile	Val	Leu	Leu	Lys	Asn	Glu	Asn	Ser	Ile	Leu	Pro	
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	Leu	Pro	Lys	Ser	Val	Lys	Lys	Val	Ala	Leu	Val	Gly	Ser	His	Val	Arg	
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	Leu	Pro	Ala	Ile	Ser	Gly	Gly	Gly	Ser	Ala	Ser	Leu	Val	Pro	Tyr	Tyr	
		210					215					220					
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	Ala	Ile	Ser	Leu	Tyr	Asp	Ala	Val	Ser	Glu	Val	Leu	Ala	Gly	Ala	Thr	
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	Ile	Thr	His	Glu	Val	Gly	Ala	Tyr	Ala	His	Gln	Met	Leu	Pro	Val	Ile	
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	Asp	Ala	Met	Ile	Ser	Asn	Ala	Val	Ile	His	Phe	Tyr	Asn	Asp	Pro	Ile	
				260					265					270			
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	Asp	Val	Lys	Asp	Arg	Lys	Leu	Leu	Gly	Ser	Glu	Asn	Val	Ser	Ser	Thr	
			275				280						285				
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	Ser	Phe	Gln	Leu	Met	Asp	Tyr	Asn	Asn	Ile	Pro	Thr	Leu	Asn	Lys	Ala	

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65	aac Asn	gag Glu	aca Thr	ggc Gly 500	cac His	gga Gly	atc Ile	tcc Ser	gat Asp 505	gtg Val	ctc Leu	ttt Phe	ggc Gly 510	aac Asn	gtc Val	aac Asn	1536	
70	ccg Pro	tcg Ser	ggg Gly 515	aaa Lys	ctc Leu	tcc Ser	cta Leu	tcg Ser 520	tgg Trp	cca Pro	gtc Val	gat Asp	gtg Val 525	aag Lys	cac His	aac Asn	1584	

	cca gca tat ctc aac tac gcc agc gtt ggt gga cgg gtc ttg tat ggc	1632
	Pro Ala Tyr Leu Asn Tyr Ala Ser Val Gly Gly Arg Val Leu Tyr Gly	
	530 535 540	
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	gag gat gtt tac gtt ggc tac aag ttc tac gac aaa acg gag agg gag	1680
	Glu Asp Val Tyr Val Gly Tyr Lys Phe Tyr Asp Lys Thr Glu Arg Glu	
	545 550 555 560	
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	gtt ctg ttt cct ttt ggg cat ggc ctg tct tac gct acc ttc aag ctc	1728
	Val Leu Phe Pro Phe Gly His Gly Leu Ser Tyr Ala Thr Phe Lys Leu	
	565 570 575	
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	cca gat tct acc gtg agg acg gtc ccc gaa acc ttc cac ccg gac cag	1776
	Pro Asp Ser Thr Val Arg Thr Val Pro Glu Thr Phe His Pro Asp Gln	
	580 585 590	
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	ccc aca gta gcc att gtc aag atc aag aac acg agc agt gtc ccg ggc	1824
	Pro Thr Val Ala Ile Val Lys Ile Lys Asn Thr Ser Ser Val Pro Gly	
	595 600 605	
25		
	gcc cag gtc ctg cag tta tac att tcg gcc cca aac tcg cct aca cat	1872
	Ala Gln Val Leu Gln Leu Tyr Ile Ser Ala Pro Asn Ser Pro Thr His	
	610 615 620	
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	cgc ccg gtc aag gag ctg cac gga ttc gaa aag gtg tat ctt gaa gct	1920
	Arg Pro Val Lys Glu Leu His Gly Phe Glu Lys Val Tyr Leu Glu Ala	
	625 630 635 640	
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	Gly Glu Glu Lys Glu Val Gln Ile Pro Ile Asp Gln Tyr Ala Thr Ser	
	645 650 655	
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	ttc tgg gac gag att gag agc atg tgg aag agc gag agg ggc att tat	2016
	Phe Trp Asp Glu Ile Glu Ser Met Trp Lys Ser Glu Arg Gly Ile Tyr	
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	Asp Val Leu Val Gly Phe Ser Ser Gln Glu Ile Ser Gly Lys Gly Lys	
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25	Leu	Arg	Thr	Glu	Trp	Asn	Trp	Asp	Pro	Leu	Ile	Val	Ser	Asp	Trp	Tyr
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30	Gly	Thr	Tyr	Thr	Thr	Ile	Asp	Ala	Ile	Lys	Ala	Gly	Leu	Asp	Leu	Glu
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40	Gln	Ala	Arg	Leu	Leu	Lys	Gln	Ser	Thr	Ile	Asp	Glu	Arg	Ala	Arg	Arg
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45	Val	Leu	Arg	Phe	Ala	Gln	Lys	Ala	Ser	His	Leu	Lys	Val	Ser	Glu	Val
	145					150					155				160	
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55	Cys	Gly	Ser	Ser	Ile	Val	Leu	Leu	Lys	Asn	Glu	Asn	Ser	Ile	Leu	Pro
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60	Leu	Pro	Lys	Ser	Val	Lys	Lys	Val	Ala	Leu	Val	Gly	Ser	His	Val	Arg
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65	Leu	Pro	Ala	Ile	Ser	Gly	Gly	Gly	Ser	Ala	Ser	Leu	Val	Pro	Tyr	Tyr
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70	Ala	Ile	Ser	Leu	Tyr	Asp	Ala	Val	Ser	Glu	Val	Leu	Ala	Gly	Ala	Thr
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Ile Thr His Glu Val Gly Ala Tyr Ala His Gln Met Leu Pro Val Ile  
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 Asp Ala Met Ile Ser Asn Ala Val Ile His Phe Tyr Asn Asp Pro Ile  
 260 265 270  
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 Asp Val Lys Asp Arg Lys Leu Leu Gly Ser Glu Asn Val Ser Ser Thr  
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 Ser Phe Gln Leu Met Asp Tyr Asn Asn Ile Pro Thr Leu Asn Lys Ala  
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 Met Phe Trp Gly Thr Leu Val Gly Glu Phe Ile Pro Thr Ala Thr Gly  
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 Ile Trp Glu Phe Gly Leu Ser Val Phe Gly Thr Ala Asp Leu Tyr Ile  
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 Asp Asn Glu Leu Val Ile Glu Asn Thr Thr His Gln Thr Arg Gly Thr  
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 Ala Phe Phe Gly Lys Gly Thr Thr Glu Lys Val Ala Thr Arg Arg Met  
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 Thr Thr Lys Met Glu Thr Thr Gly Val Val Asn Phe Gly Gly Gly Ala  
 385 390 395 400  
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 Val His Leu Gly Ala Cys Leu Lys Val Asp Pro Gln Glu Met Ile Ala  
 405 410 415  
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 Arg Ala Val Lys Ala Ala Ala Asp Ala Asp Tyr Thr Ile Ile Cys Thr  
 420 425 430  
 Gly Leu Ser Gly Glu Trp Glu Ser Glu Gly Phe Asp Arg Pro His Met  
 435 440 445  
 Asp Leu Pro Pro Gly Val Asp Thr Met Ile Ser Gln Val Leu Asp Ala  
 450 455 460

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5	Ser	Trp	Ala	His	Lys	Ala	Lys	Ala	Ile	Val	Gln	Ala	Trp	Tyr	Gly	Gly
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	625					630					635					640
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&lt;220&gt;

&lt;223&gt; synthetic ferulic acid esterase

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45

&lt;210&gt; 100

&lt;211&gt; 395

&lt;212&gt; PRT

50 &lt;213&gt; artificial sequence

&lt;220&gt;

&lt;223&gt; synthetic ferulic acid esterase

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10	Thr Ala Thr Asn Ser Thr Arg Pro Ala Arg Val Tyr Leu Pro Pro Gly	35	40	45
15	Tyr Ser Lys Asp Lys Lys Tyr Ser Val Leu Tyr Leu Leu His Gly Ile	50	55	60
20	Gly Gly Ser Glu Asn Asp Trp Phe Glu Gly Gly Gly Arg Ala Asn Val	65	70	75
25	Ile Ala Asp Asn Leu Ile Ala Glu Gly Lys Ile Lys Pro Leu Ile Ile	85	90	95
30	Val Thr Pro Asn Thr Asn Ala Ala Gly Pro Gly Ile Ala Asp Gly Tyr	100	105	110
35	Glu Asn Phe Thr Lys Asp Leu Leu Asn Ser Leu Ile Pro Tyr Ile Glu	115	120	125
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50	Asp Lys Phe Ala Tyr Ile Gly Pro Ile Ser Ala Ala Pro Asn Thr Tyr	165	170	175
55	Pro Asn Glu Arg Leu Phe Pro Asp Gly Gly Lys Ala Ala Arg Glu Lys	180	185	190
60	Leu Lys Leu Leu Phe Ile Ala Cys Gly Thr Asn Asp Ser Leu Ile Gly	195	200	205
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 30 Val Lys Ser Thr Gly Asp Trp Asn Thr Tyr Glu Glu Gln Thr Cys Ser  
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Thr Ala Thr Asn Ser Thr Arg Pro Ala Arg Val Tyr Leu Pro Pro Gly  
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25	Val Lys Ser Thr Gly Asp Trp Asn Thr Tyr Glu Glu Gln Thr Cys Ser			
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	225					230					235				240	
50	Ile	Asn	His	Val	Tyr	Trp	Leu	Ile	Gln	Gly	Gly	Gly	His	Asp	Phe	Asn
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Thr Cys Ser Ile Ser Lys Val Thr Gly Ile Asn Asp Leu Tyr Leu Val  
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 25 accccggagg tggcccgtc ccgcgccacc ccgctcctcg acctcatcaa gaccgccctc 900  
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			35					40					45			
10	Leu	Met	Gln	Asp	Val	Thr	Pro	Asp	Ala	Trp	Pro	Thr	Trp	Pro	Val	Lys
		50					55					60				
15	Leu	Gly	Glu	Leu	Thr	Pro	Arg	Gly	Gly	Glu	Leu	Ile	Ala	Tyr	Leu	Gly
	65					70					75					80
20	Gly	Cys	Pro	Gln	Ser	Gly	Gln	Val	Ala	Ile	Ile	Ala	Asp	Val	Asp	Glu
				100					105					110		
25	Arg	Thr	Arg	Lys	Thr	Gly	Glu	Ala	Phe	Ala	Ala	Gly	Leu	Ala	Pro	Asp
			115					120					125			
30	Cys	Ala	Ile	Thr	Val	His	Thr	Gln	Ala	Asp	Thr	Ser	Ser	Pro	Asp	Pro
		130					135					140				
35	Leu	Phe	Asn	Pro	Leu	Lys	Thr	Gly	Val	Cys	Gln	Leu	Asp	Asn	Ala	Asn
	145					150					155					160
40	Val	Thr	Asp	Ala	Ile	Leu	Glu	Arg	Ala	Gly	Gly	Ser	Ile	Ala	Asp	Phe
					165					170					175	
45	Thr	Gly	His	Tyr	Gln	Thr	Ala	Phe	Arg	Glu	Leu	Glu	Arg	Val	Leu	Asn
				180					185					190		
50	Phe	Pro	Gln	Ser	Asn	Leu	Cys	Leu	Lys	Arg	Glu	Lys	Gln	Asp	Glu	Ser
			195					200					205			
55	Cys	Ser	Leu	Thr	Gln	Ala	Leu	Pro	Ser	Glu	Leu	Lys	Val	Ser	Ala	Asp
		210					215					220				
60	Cys	Val	Ser	Leu	Thr	Gly	Ala	Val	Ser	Leu	Ala	Ser	Met	Leu	Thr	Glu
	225					230					235					240
65	Ile	Phe	Leu	Leu	Gln	Gln	Ala	Gln	Gly	Met	Pro	Glu	Pro	Gly	Trp	Gly
				245						250					255	

Arg Ile Thr Asp Ser His Gln Trp Asn Thr Leu Leu Ser Leu His Asn  
 260 265 270

5

Ala Gln Phe Asp Leu Leu Gln Arg Thr Pro Glu Val Ala Arg Ser Arg  
 275 280 285

10

Ala Thr Pro Leu Leu Asp Leu Ile Lys Thr Ala Leu Thr Pro His Pro  
 290 295 300

15

Pro Gln Lys Gln Ala Tyr Gly Val Thr Leu Pro Thr Ser Val Leu Phe  
 305 310 315 320

20

Ile Ala Gly His Asp Thr Asn Leu Ala Asn Leu Gly Gly Ala Leu Glu  
 325 330 335

Leu Asn Trp Thr Leu Pro Gly Gln Pro Asp Asn Thr Pro Pro Gly Gly  
 340 345 350

25

Glu Leu Val Phe Glu Arg Trp Arg Arg Leu Ser Asp Asn Ser Gln Trp  
 355 360 365

30

Ile Gln Val Ser Leu Val Phe Gln Thr Leu Gln Gln Met Arg Asp Lys  
 370 375 380

35

Thr Pro Leu Ser Leu Asn Thr Pro Pro Gly Glu Val Lys Leu Thr Leu  
 385 390 395 400

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Ala Gly Cys Glu Glu Arg Asn Ala Gln Gly Met Cys Ser Leu Ala Gly  
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Phe Thr Gln Ile Val Asn Glu Ala Arg Ile Pro Ala Cys Ser Leu  
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5 cgcgccccga ccaaggccac ccagctcatg caggacgtga ccccggaacgc ctggccgacc 180  
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 Ala Thr Ser Ala Ala Gln Ser Glu Pro Glu Leu Lys Leu Glu Ser Val

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5	Val Ile Val Ser Arg His Gly	Val Arg Ala Pro Thr Lys	Ala Thr Gln
	35	40	45
10	Leu Met Gln Asp Val Thr Pro Asp Ala Trp Pro Thr Trp Pro Val Lys		
	50	55	60
15	Leu Gly Glu Leu Thr Pro Arg Gly Gly Glu Leu Ile Ala Tyr Leu Gly		
	65	70	75
20	His Tyr Trp Arg Gln Arg Leu Val Ala Asp Gly Leu Leu Pro Lys Cys		
	85	90	95
25	Gly Cys Pro Gln Ser Gly Gln Val Ala Ile Ile Ala Asp Val Asp Glu		
	100	105	110
30	Arg Thr Arg Lys Thr Gly Glu Ala Phe Ala Ala Gly Leu Ala Pro Asp		
	115	120	125
35	Cys Ala Ile Thr Val His Thr Gln Ala Asp Thr Ser Ser Pro Asp Pro		
	130	135	140
40	Leu Phe Asn Pro Leu Lys Thr Gly Val Cys Gln Leu Asp Asn Ala Asn		
	145	150	155
45	Val Thr Asp Ala Ile Leu Glu Arg Ala Gly Gly Ser Ile Ala Asp Phe		
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50	Thr Gly His Tyr Gln Thr Ala Phe Arg Glu Leu Glu Arg Val Leu Asn		
	180	185	190
55	Phe Pro Gln Ser Asn Leu Cys Leu Lys Arg Glu Lys Gln Asp Glu Ser		
	195	200	205
60	Cys Ser Leu Thr Gln Ala Leu Pro Ser Glu Leu Lys Val Ser Ala Asp		
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65	Cys Val Ser Leu Thr Gly Ala Val Ser Leu Ala Ser Met Leu Thr Glu		
	225	230	235
70	Ile Phe Leu Leu Gln Gln Ala Gln Gly Met Pro Glu Pro Gly Trp Gly		
	245	250	255

Arg Ile Thr Asp Ser His Gln Trp Asn Thr Leu Leu Ser Leu His Asn  
260 265 270  
5  
Ala Gln Phe Asp Leu Leu Gln Arg Thr Pro Glu Val Ala Arg Ser Arg  
275 280 285  
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Ala Thr Pro Leu Leu Asp Leu Ile Lys Thr Ala Leu Thr Pro His Pro  
290 295 300  
15  
Pro Gln Lys Gln Ala Tyr Gly Val Thr Leu Pro Thr Ser Val Leu Phe  
305 310 315 320  
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Ile Ala Gly His Asp Thr Asn Leu Ala Asn Leu Gly Gly Ala Leu Glu  
325 330 335  
25  
Leu Asn Trp Thr Leu Pro Gly Gln Pro Asp Asn Thr Pro Pro Gly Gly  
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30  
Glu Leu Val Phe Glu Arg Trp Arg Arg Leu Ser Asp Asn Ser Gln Trp  
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Ile Gln Val Ser Leu Val Phe Gln Thr Leu Gln Gln Met Arg Asp Lys  
370 375 380  
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Thr Pro Leu Ser Leu Asn Thr Pro Pro Gly Glu Val Lys Leu Thr Leu  
385 390 395 400  
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Ala Gly Cys Glu Glu Arg Asn Ala Gln Gly Met Cys Ser Leu Ala Gly  
405 410 415  
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Phe Thr Gln Ile Val Asn Glu Ala Arg Ile Pro Ala Cys Ser Leu Ser  
420 425 430  
Glu Lys Asp Glu Leu  
435